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Selenium and human health

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Selenium and human health

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Editorial: Selenium and human health

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Editorial on the Research Topic
Selenium and human health

Selenium (Se) is an indispensible trace element to human health. Due to the uneven distribution of selenium around the world, it leads to insufficient or excessive Se intake among residents in some regions (1). The World Health Organization (WHO) recommends the intake of Se ranged from 50 to 200 μg/d/adult (2). Notably, the impact of Se on human health is not only related to the intake of Se, but also closely related to the type and valence of Se (3–5). Currently, it has been proved that more than 40 diseases are highly related to Se deficiency (6, 7), such as Keshan disease, cataract, diabetes, thyroid disease, cancer, etc. (Cai et al.). However, excessive Se intake can also lead to skin damage, nail and hair loss, gastrointestinal discomfort, neurological damage, paralysis, and even death (8). Therefore, Se plays a vital role in human health. In the face of issues such as insufficient or excessive Se intake caused by the uneven distribution of Se in geographical locations, it is of great significance to further understand the latest research advances in Se and human health.

The relationship between gut microbiota and health is an emerging research hotspot recently. Cai et al. reviewed the regulatory mechanism of Se on human gut microbiota and its impact on diseases. They concluded that insufficient Se intake might lead to changes in the phenotype of intestinal microbiota, making humans more vulnerable to inflammatory disease, thyroid disease, cancer, etc. Meanwhile, the authors also pointed out shortcomings of the current research and the future research directions: (i) relationship between the optimal daily Se intake of human beings and the Se demand of normal gut microbiota needs further research; (ii) more clinical studies are needed to prove the relationship between human gut microbiota and occurrence of diseases; (iii) the combination of high-throughput sequencing and multi-omics can solve the difficulties in the research of effect of Se on gut microbiota.

The long-term influence of serum Se level on the health of cardiovascular patients with chronic kidney disease is unclear till now. Zhu et al. from Sichuan University investigated the correlation between serum Se levels and all-cause /cardiovascular disease (CVD) mortality in 3,063 CVD patients with chronic kidney disease (CKD), and estimated the hazard ratios (HRs) and 95% CI using Cox professional hazards model and competitive risk model. Results indicated that 884 deaths occurred including 336 CVD-associated deaths, the median (IQR) concentration of serum Se of the deaths was 181.7 (156.1–201.5) μg/L. After Se supplement, the mortality of CVD patients with CKD including CVD and all-cause mortality decreased with the increase of serum Se level ($p < 0.001$). The multivariate-adjusted HRs (95% CI) were 0.513 (0.356–0.739) for CVD mortality ($p_{trend} < 0.001$) and 0.684 (0.549–0.852) for all-cause mortality ($p_{trend} < 0.001$) when Se levels were compared to the extreme quartiles, suggesting that higher serum Se level was associated with lower risk of CVD and all-cause mortality among CVD patients with CKD.

Chen X. et al. investigated the protective effect of different Se supplements on liver injury caused by heat stress and exercise fatigue and the related mechanisms. SD rats were continuously gavaged with Se-rich soy protein (SePro), Na_2SeO_3, common peptides (PPs), Se-rich soy peptides (SePPs) and selenomethionine (SeMet) for 7 days. Results showed that the levels of catalase, superoxide dismutase, malondialdehyde in serum and activities of GSH and GSH-Px in liver of SePPs rats were significantly different from those in the control group ($p < 0.05$), SePPs significantly increased the antioxidant level of SD rats, inhibited lipid peroxidation and reduced the activities of liver enzymes of SD rats. Histological studies found that the infiltration of inflammatory cells in the liver tissue of SePPs rats decreased and new cells appeared in livers of SePPs rats. In addition, SePPs increased glutathione content and GSH peroxidase activity in rat liver, and protected rat liver by regulating NF-κB/IκB pathway and preventing the release of interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α). SePPs showed better liver protection effects than SePro, SeMet, Na_2SeO_3 and PPs. Therefore, SePPs can be used as a priority Se resource to develop supplement to prevent liver damage caused by heat stress and exercise fatigue.

Colon carcinoma is one of the serious malignant tumors, and its prevention and treatment have been a hot Research Topic in the medical field. Gao et al. prepared stable SeNP using lentinan (LNT) as a template, and evaluated its inhibitory activity on colon carcinoma. The average particle size of obtained LNT-SeNP was about 59 nm, which was highly stable at 4°C for over 8 weeks, and maintained zero valence, amorphous or spherical structure. LNT stabilized SeNP through hydrogen bond interaction. LNT-SeNP had no significant cytotoxicity on normal cells (IEC-6), but significantly inhibited the proliferation of five types of colon carcinoma cells (HCT-116, HT-29, Caco-2, SW620, and CT26). Among them, LNT-SeNP had the highest sensitivity to HCT-116 cells with an IC_{50} value of 7.65 μM. In addition, LNT-SeNP promoted apoptosis of HCT-116 cells by activating mitochondrial mediated apoptosis pathways. At the same time, LNT-SeNPs induced arrest of development in HCT-116 cells during G0/G1 phase by regulating cell cycle regulatory proteins. These results indicate that LNT-SeNP has a promising application prospect in the treatment of colon carcinoma.

Selenium is capable of regulating autophagy through different signaling pathways, playing an important role in maintaining normal cellular physiological functions and protecting organisms. SeNPs exhibit better biological activity compared to inorganic Se, and research on the regulatory effects of SeNPs on cellular signaling pathways is gradually increasing. Chen D. et al. reviewed the latest research progress on SeNPs inhibiting cancer cell growth or inducing cancer cell death by promoting autophagy in cancer cells. Numerous studies showed that SeNPs enhanced the autophagy ability of cancer cells by activating the ROS mediated JNK pathway and inhibiting the PI3K/Akt/mTOR pathway, achieving better anticancer effects. SeNPs were also used to treat cancer by combination with current chemotherapy methods, achieving better therapeutic effects. In addition, Chen D. et al. summarized the current research advance of SeNPs in reducing drug toxicity, regulating inflammatory response, resisting pathogen infection and treating Alzheimer's disease by regulating autophagy. This review enables us to recognize that SeNPs can serve as a novel autophagy regulator, providing new possibilities for the clinical treatment of some important diseases (Chen D. et al.).

Selenoproteins relates to glucose metabolism or insulin resistance, but the relationship between serum Se level and type 2 diabetes is uncertain. Zhao et al. reviewed the latest research advance in the regulation of Se on metabolism and type 2 diabetes. Results showed that Se supply was very important for maintaining health and glucose and lipid homeostasis in type 2 diabetes patients. Epidemiological analysis of blood Se level in type 2 diabetes patients showed that both Se deficiency and severe excess led to insulin resistance and β cell dysfunction. The potential molecular mechanisms of β cell dysfunction included oxidative stress, insulin signal transduction, interference of gluconeogenesis and endoplasmic reticulum stress. In this regard, Se supplementation requires personalized nutritional recommendations, which needs to consider the regional characteristics and genetic characteristics of individual (Zhao et al.).

In addition to the concentration of Se, the form, valence state and source of Se also have a significant impact on its bioactivity. Tangjaidee et al. introduced the enrichment capacity and forms of Se in Se-rich plant food, the biological effects of Se and Se-containing compounds on human health, and the prospect of Se-rich plant food. The author believed that the biological characteristics of Se-rich plant food were closely related to the chemical form and Se content. Selenium-rich plant food with non-toxic concentration could provide health benefits by increasing the antioxidant activity in human serum. Other biological characteristics, such as antioxidant activity, anti-diabetes and anti-cancer activities, were also demonstrated *in vitro* cell culture models and *in vivo* animal experiments. In contrast, there are still relatively few available data of clinical trials about the effect of Se-rich plant food on human health, thus it is crucial to obtain valuable information supporting the beneficial effect of Se-rich food on human body.

It is an important application of Se to enhance the growth performance, disease resistance and meat quality of poultry through Se supplementation in feed. Xu et al. explored the effects of feeds containing sodium selenite (SeNa), Se yeast (SeY) and Se-rich *Cardamine violifolia* (SeCv) on the growth performance, antioxidant capacity and meat quality of broilers. Results indicated that the broilers fed with SeCv enhanced the average daily gain and ratio of feed to gain compared to those of the broilers fed with SeNa and SeY during the earlier stage (the earlier 21 days). Meanwhile, the total liver anti-oxidative capacity of broilers fed with SeCv was enhanced compared with those of broilers fed with the other two Se sources. In the later stage (from days 21 to 42), the intestinal mucosal morphology of broilers fed with SeCv was improved, and lower liver malondialdehyde contents in the broilers fed with SeCv and SeY were found when compared with that of broilers fed with SeNa. As for the meat quality, SeCv increased the redness of thigh muscle and decreased the cooking loss in both breast and thigh muscle of the boilers compared with those of the broilers fed with SeNa. The above investigation demonstrated that SeCv feed might be a potential Se source to improve the growth performance, antioxidant capacity and meat quality of poultry.

Nano-selenium (nano-Se) was generally regarded as a biostimulant for plants. However, the specific effect on the Chinese herbal medicine was scarcely reported. Dong et al. sprayed nano-Se on the leaves of *Panax notoginseng* (SePN) during its growth. Compared with the control (PN), it was found that spraying nano-Se on the leaves increased the saponin contents (Rb2, Rb3, Rc, F2, Rb2, and Rf) in root of *Panax notoginseng*, of them, a significant increase of 3.9-time of Rb2 content was observed. Animal study showed that the liver malondialdehyde content of mice taking SePN decreased significantly ($p < 0.01$) compared with the control group, and its glutathione content increased significantly ($p < 0.05$). Meanwhile, it increased the enzyme activities involved in glycolipid metabolism (ATGL and PFK), alleviated inflammation and regulated genes related to fatty acid oxidation (MCAD, PPAR-α, and PCSK9) expression of mice. It can be concluded that nano-Se bioaugmentation improved the bioactivity of *Panax notoginseng*.

Soy sauce is rich in free amino acids, low molecular weight peptides and phenolic substances, which have strong antioxidant activity, making it a good carrier of Se. Chen J. et al. added nano-Se during soybean soaking and investigated the bioconversion activity of soybean on Se and the effect of nano-Se on soy sauce quality. Results showed that the total and organic Se contents of soy sauces increased by 32–191 times compared with those of the control, of them, organic Se content accounted for more than 90% of the total Se in soy sauce. Soy sauce prepared by soaking soybeans in 6 mg/L nano-Se solution had the strongest antioxidant activity, which was 9.25–28.02% higher than the control. Nano-Se (6 mg/L) significantly increased the enzyme activities of Daqu (9.76–33.59%), promoted the release of total phenols (27.54%), total flavonoids (27.27%) in soybeans, and the formation of free amino acids (16.19%) and Maillard reaction products (24.50%) in soy sauce. The above method not only significantly increased the organic Se content in soy sauce, but also improved the antioxidant activity of soy sauce, indicating that nano-Se has a broader application prospect in Se-rich fermented foods.

This Research Topic provides valuable information about the latest research on Se and human health. We hope the Research Topic can contribute to scientific advance, more importantly, make people aware of the importance and complexity of the relationship between Se and human health. To the best of our knowledge, the following aspects are the future research hotspots: (1) More extensive epidemiological study on serum Se content and human health, (2) The effect of different valence states of Se (inorganic Se, organic Se and nano-Se) on diseases caused by selenium deficiency and the related mechanism, (3) Development of Se-rich foods, and the effect of Se-rich foods on human health and the related mechanism.

Author contributions

XG: Data curation, Funding acquisition, Supervision, Validation, Writing—original draft, Writing—review and editing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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A Comprehensive Comparison of Different Selenium Supplements: Mitigation of Heat Stress and Exercise Fatigue-Induced Liver Injury

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This study aimed to compare the protective effects of different selenium supplements against heat stress and exercise fatigue-induced liver injury and to investigate the potential mechanisms of action. Selenium-enriched soybean protein (SePro), selenium-enriched soybean peptides (SePPs), and selenomethionine (SeMet) are organic selenium supplements in which selenium replaces the sulfur in their sulfur-containing amino acids. Common peptides (PPs) are obtained by enzymatic hydrolysis of soybean protein which was extracted from common soybean. The SePPs with higher hydrolysis degree and selenium retention were isolated *via* alkaline solubilization and acid precipitation and the enzymatic hydrolysis of alkaline protease, neutral protease, and papain. The results showed that SePPs could significantly increase the antioxidant levels in rats, inhibit lipid peroxidation, and reduce liver enzyme levels in rat serum, while the histological findings indicated that the inflammatory cell infiltration in the liver tissue was reduced, and new cells appeared after treatment with SePPs. Moreover, SePPs could increase glutathione (GSH) and GSH peroxidase (GSH-Px) in the liver, as well as protect the liver by regulating the NF-κB/IκB pathway, prevent interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) release in the liver. The SePPs displayed higher antioxidant and anti-inflammatory activity *in vivo* than SePro, SeMet, Sodium selenite (Na_2SeO_3), and PPs. Therefore, SePPs could be used as a priority selenium resource to develop heatstroke prevention products or nutritional supplements.

Keywords: selenium-enriched soybean peptides, heat stress, exercise fatigue, heatstroke, oxidative damage, inflammation

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INTRODUCTION

In recent years, with the general increase in the concept of health, more and more people are running, hiking, and doing other sports to promote their health. Meanwhile, some occupational groups (such as athletes, workers, etc.) require higher exercise intensity, which increases the incidence of exercise-related diseases. Therefore, sports health has drawn increasing attention during the past decades. Heatstroke is one of the common sports injury-related diseases, mainly caused by heat stress and exercise fatigue. Hyperthermic exercise is associated with severe fluid loss, which leads to the formation of reactive oxygen species (ROS) and affects the body's

redox balance (1). ROS can disrupt cell membranes and allow endotoxins and pathogens to leak into the human circulation, resulting in the destruction of the liver detoxification function and accompanying inflammatory response, eventually causing hepatocyte necrosis or liver tissue damage (2). Electrolyte or water supplementation alone has limited recovery from heatstroke induced inflammatory response and tissue damage. The most important treatment for patients with heatstroke is rapid and effective body cooling, while the endpoint of the cooling is controversial. The controversial lies in whether to stop cooling by targeting the patient's core body temperature or behavioral performance (3). Consequently, developing functional foods or heatstroke preventive drinks has become a crucial and practical strategy to avoid heatstroke.

Selenium is an essential trace element for the body and a key component of the GSH-Px. GSH-Px is expressed in the cytoplasm and mitochondria, which can enhance the antioxidant capacity of the body and protect the structure and function of cells (4). The functions of selenium have been demonstrated previously, including detoxification, antioxidant and immune enhancement. It was reported that low selenium consumption was linked to various human disorders. Zhang et al. found that selenium deficiency disrupted the normal membrane structure of spleen cells, downregulated the expression levels of immune response-related genes, and activated T lymphocyte differentiation *via* the DUSP1/NF-κB pathway, affecting the immune response in the spleen and causing tissue damage (5). Selenium is an element that cannot be synthesized by the human body and needs to be consumed from food. Selenium supplements are classified as inorganic selenium (selenates and selenites) and organic selenium (selenocysteine [SeCys], selenomethionine [SeMet], selenoproteins and their hydrolysates) (6). Nevertheless, selenium is considered both beneficial and toxic depending on its form and amount ingested (7). The organic form of selenium is superior to the inorganic form in terms of bioavailability and toxicity for meeting human dietary requirements (8). Consequently, it is necessary to explore new selenium carriers to address the potential problems associated with selenium deficiency and selenium toxicity.

Soybean protein has a high nutritional value and contains a complete range of amino acids and eight essential amino acids (9). More importantly, soybean exhibits a high selenium enrichment capacity, with more than 75% of selenium in soybean bound to protein. Up to 82% of selenium is present in high molecular weight form, primarily in SeCys and SeMet (10). The bioavailability of selenium from soy protein can be as high as 86-96% (11). Previous results have shown that selenium-enriched soy protein hydrolysates are healthy nutrients. According to Liu et al., selenium biofortified soy peptides could reduce CCl_4-induced liver fibrosis by increasing GSH-Px activity and GSH content, improving hepatocyte survival, inhibiting hepatic stellate cell activation, and improving antioxidant capacity (12). Zhang et al. observed that SePPs could regulate immunoglobulin (Ig)M, IgG, and IgA secretion, significantly increase splenic interleukin 2 (IL-2), interferon-gamma (IFN-γ), nitric oxide (NO), and cyclic guanosine monophosphate (cGMP) production, and IL-2, IFN-γ, and inducible NO synthase (iNOs) mRNA expression, as well as enhance host-specific and non-specific immunity *via* a cyclophosphamide-induced immunosuppression mice model (13). The beneficial effects of selenium-enriched peptides on health were confirmed previously, whereas, the protective mechanism of selenium-enriched peptides against heatstroke-induced liver injury is still unclear. Meanwhile few studies attempted to compare the effects of different selenium supplements.

This study, isolated SePro from naturally grown selenium-enriched soybeans, which were enzymatically separated to produce SePPs. A modeling approach of rats swimming in hot water under heat stress and exercise fatigue-induced heatstroke was used to investigate the protective effect of SePPs against sports injury and the possible mechanisms of action. The impact of different forms of selenium supplements was compared comprehensively. It was predicted that SePPs would prevent heatstroke and increase exercise tolerance by reducing oxidative stress and regulating inflammatory responses. This study provides a basis for preventing sports diseases and also provides new ideas for selenium nutrition supplementation.

MATERIALS AND METHODS

Materials and Chemicals

Selenium-enriched soybeans were purchased from Enshi Se-Run Health Tech Development Co., Ltd. (Enshi city, China), while L-SeMet was obtained from Shanghai Macklin Biochemical Co., Ltd. Na_2SeO_3 was purchased from Sigma Chemical Co. (St. Louis, MO, United States). Xi'an Ruidi Biotechnology Co., Ltd., supplied the honeysuckle extract. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), GSH, and GSH-Px kits were obtained from Nanjing Jiancheng Bioengineering Institute. The IL-1β, IL-6, TNF-α, GSH, and GSH-Px ELISA kits were acquired from Jiangsu Meibiao Biotechnology Co., Ltd., while the BCA protein concentration assay kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. The skim milk powder was supplied by Becton, Dickinson, and Company. Furthermore, the PVDF membrane, TBST, NF-κB p65 rabbit monoclonal antibody, phospho-IκBα (Ser32/36) rabbit polyclonal antibody, α-Tubulin rabbit polyclonal antibody, horseradish peroxidase-labeled goat anti-rabbit IgG (H + L), Western secondary antibody dilution, and BeyoECL Star (extra super-sensitive enhanced chemiluminescence kit) were purchased from Beyotime Biotechnology.

Preparation of SePPs

The SePPs was prepared as described in a previous study (14). The selenium-enriched soybeans were crushed, defatted, and dried to obtain a powder, after which the SePro was extracted *via* alkaline solubilization and acid deposition. The preparation process is shown in **Figure 1**. Alkaline solubilization was performed at 40°C for 2 h at a 1:20 mater-liquid ratio and pH = 8. The SePro was obtained *via* centrifugation at 2,100 g for 20 min, after which the supernatant was adjusted

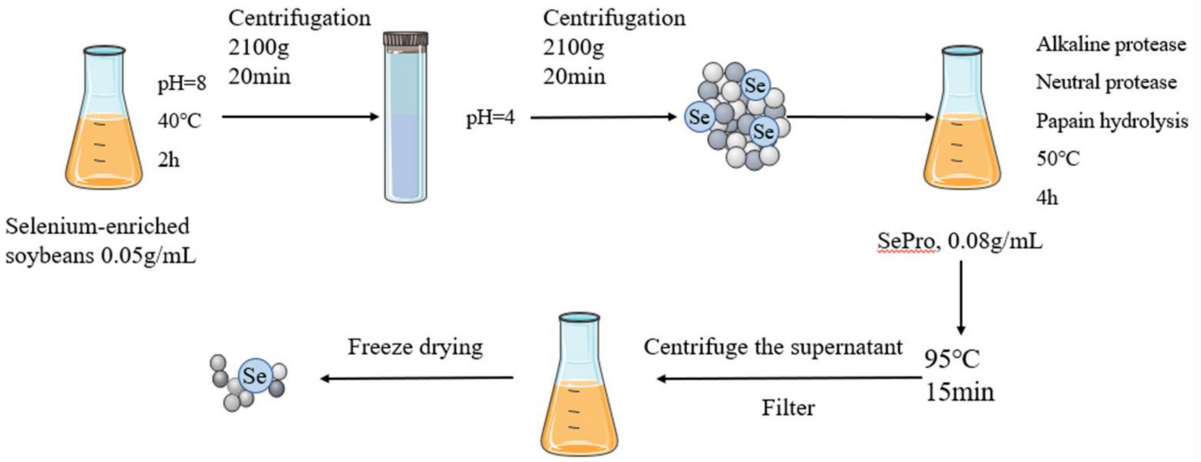

FIGURE 1 | Preparation of SePPs.

to pH 4.0 and centrifuged at 2,100 g for 20 min. Subsequently, the SePro was dissolved in water to obtain a SePro solution with a mass fraction of 8%, using alkaline protease, neutral protease, and papain. Enzymatic digestion occurred at 50°C for 4 h a ratio of 2:1:1. Finally, the enzymatic digest was heated at 95°C for 15 min to inactivate the protease, to ensure that the degree of hydrolysis reached 68%. After centrifugation, the supernatant was collected and filtered through a 0.45 μm microporous membrane. SePPs were obtained after freeze-drying.

Protein content, selenium content and amino acid composition were determined as described in previous reports (14). The protein content in the SePro, SePPs, and common soy peptides (PPs) was assessed using the Kjeldahl method (Kjeltec 8000, FOSS analysis A/S) at a conversion factor setting of 6.25. The total selenium content in the solid SePro, SePPs and PPs powders was measured using hydride generation-atomic fluorescence spectrometry (LC-AFS6500, Beijing Haiguang Instruments Co., Ltd.). The amino acid composition of SePro, SePPs, and PPs was assessed using an amino acid analyzer (Biochrom 30 amino acid analyzer; Biochrom Ltd.) and a Na cation exchange column (8 mm, 4.6 × 200 mm), which was purchased from the Waters Corporation. The amino acids were derivatized with ninhydrin reagent after they passed through the exchange column. The absorbance of the subsequent compounds was measured at 440 nm (proline [Pro]) and 570 nm (all other amino acids). The molecular weight distribution of SePPs was determined following a previously reported method (13). Standard molecular weight samples of aprotinin (6500 Da), bacitracin (1422 Da), Gly-Gly-Tyr-Arg (451 Da), and reduced GSH (307 Da) were passed through a 0.22 μm filter and deposited in a Superdex 200 10/300 GL column. The chromatographic analysis was performed using an ÄKTA pure system (AKTA pure 25, Cytiva). The phosphate buffer (PBS, concentration 0.05 M, pH = 7) mobile phase was eluted at a 0.5 ml/min flow rate and detected at 220 nm.

Animals and Diet

Eighty male Sprague-Dawley (SD) rats (6 weeks old, weight 170 ± 10 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., license No. SCXK (Beijing) 2016-0006. The rats received a standard diet and were provided free access to water. They were kept at a temperature of 22 ± 2°C, a humidity of 50 ± 10%, and a 12 h light/12 h dark cycle. The rats were acclimatized for one week before the experiment. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Pony Testing International Group Co., Ltd. (PONY-2021-FL-15).

Establishing the Heatstroke Rat Model

The 80 male SD rats were randomly divided into eight groups (n = 10): control group, model group, positive control (PC) honeysuckle group (15), SePro group, SePPs group, PPs group, SeMet group, and Na_2SeO_3 group. All rats were administered *via* gavage, while the control and model groups were given equal volumes of deionized water, in which the selenium content 18 μg/kg in selenium-containing groups was equal, equivalent to 3.57 μg/kg in adults (Reference Chinese Nutrition Association: The recommended adult daily intake of selenium is 50-250 μg, 200 μg was selected for conversion). The protein composition of the PPs group was the same as that of SePPs group, and the rats were gavaged once daily for 7 d. The last gavage was administered after a 12-h fasting period. After 10 min of gavage, the rats in the control group were kept at room temperature (22 ± 2°C), while the rats in the model group were placed in hot water to swim at 40 ± 2°C to observe their status. When the activity of the rats decreased, the hot water near them was agitated with a wooden stick to keep them moving continuously. The rats were removed immediately when they started to sink, and the time was recorded. The remaining groups of rats swam in hot water for the same duration as the model group. After completing the swimming process, the rats were anesthetized with 3% sodium pentobarbital and dissected

to collect the material. Blood was taken from the abdominal aorta, serum was separated for blood indicator analysis, and liver tissue was collected for histomorphometric analysis, while the rest was flash-frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Determination of the Oxidative Stress Indicators in the Serum

The SOD, CAT, GSH-Px, MDA, and GSH levels in serum were measured using commercial kits according to the instructions of the manufacturer.

Determination of Serum Liver Enzyme Levels

The ALT and AST viability in serum were measured using commercial kits according to the instructions of the manufacturer. The optical density (OD) values of the solutions were measured at 510 nm using an automated microplate reader (Infinite 200 Pro Nanoquant, Tecan).

Determination of the GSH and GSH-Px Levels in the Liver Tissues

Tissue samples were collected and rinsed with saline. The filter paper was blotted to dry the surrounding water, after which the tissues were cut, weighed, and placed on ice with a delay of 5 s after every 5 s of grinding. The grinding process was repeated five times for tissue homogenization, and the tissue homogenate used PBS (pH = 7.2-7.4, concentration of 0.01 mol/L) with a homogenization ratio of 10%. The supernatant was collected for analysis after centrifugation at 2296 g for 15 min. The GSH and GSH-Px levels in liver tissue were measured using a commercial rat ELISA quantification kit according to the instructions of the manufacturer. The OD values of the solutions were measured at 450 nm using an automated microplate reader (Infinite 200 Pro Nanoquant, Tecan).

Histological Analysis

Some of the liver tissue was immediately fixed in formalin for 48 h, embedded in paraffin, stained with hematoxylin and eosin, and connected to a microscope for observation and morphological analysis, which was used to assess the degree of liver tissue damage.

Determination of the Inflammatory Factors in Liver Tissue

The liver tissue was pre-treated as described in Section 2.7. The IL-1β, IL-6, and TNF-α concentrations in the liver tissue were measured using commercial rat ELISA quantification kits according to the instructions of the manufacturer. The OD values of the solutions were measured at 450 nm using an automated microplate reader (Infinite 200 Pro Nanoquant, Tecan).

Western Blot

The liver samples were added to the lysate (RIPA) with phosphatase inhibitors, placed on ice and ground, and delayed for 5 s after every 5 s of grinding. The grinding process was repeated five times, after which the extracts were centrifuged for 4 min at 12000 g. The protein concentration was determined by adding diluent to the BCA supernatant to unify the protein concentrations of all the samples. Then 5 × loading buffer was added and placed in a 100°C water bath. The mixture was heated for 5 min for denaturing, cooled, and centrifuged at 9184 g for 2 min, after which the supernatant was collected for determination. The protein was separated in 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skim milk, the primary antibody (NF-κB p65 rabbit monoclonal antibody, phospho-IκBα (Ser32/36) rabbit polyclonal antibody, 1:1000) was incubated overnight at 4°C, after which the primary antibody was washed off. The secondary antibody [anti-rabbit IgG(H + L) 1:1000] was incubated for 1 h at room temperature, and protein expression was normalized with α-Tubulin *via* ECL chemiluminescence. Band intensities were measured using Image J software.

Statistical Analysis

The data were expressed as mean ± standard deviation (SD). The results were analyzed *via* one-way analysis of variance (ANOVA) and Tukey's method using SPSS 23 software. A P value less than 0.05 indicated statistical significance.

RESULTS

Characterization of SePPs

The protein contents of the SePro, SePPs, and PPs were 89.36 ± 0.24%, 84.46 ± 0.03%, and 88.98 ± 0.15%, respectively, while the total selenium contents in the solid SePro, SePPs, and PPs powders were 44.00 ± 0.95 mg/kg, 26.00 ± 0.7 mg/kg and 0.06 ± 0.001 mg/kg, respectively. During alkaline solubilization and acid deposition, the proteins were separated *via* acid-base washing and dialyzed to remove the small molecules (including inorganic selenium not attached to the proteins), leaving only organic selenium in SePro and SePPs. **Table 1** showed the amino acid compositions of the SePro, SePPs, and PPs. SePro, SePPs, and PPs contained a comprehensive variety of amino acids, including eight essential amino acids, as well as aliphatic, aromatic, and basic amino acids.

As shown in **Table 2**, more than 90% of the components of SePPs and PPs have molecular weights less than 3000 Da. The molecular weight of peptides represents a vital influencing factor of their functional activity, while bioactive peptides are typically a mixture of protein hydrolysis products and low molecular weight peptides.

Effects of Different Selenium Supplements on the Oxidative Damage in the Heatstroke Rats *in vivo*

When the rats were removed from the hot water, their eyes were dull and bulging, while most were not moving, indicating the occurrence of heatstroke. The CAT, SOD, GSH-Px, MDA, and GSH levels in the serum were measured to evaluate the

TABLE 1 | The amino acid compositions (%) in SePro, SePPs, and PPs.

	SePro (%)	SePPs (%)	PPs (%)
Asp	10.63 ± 0.02	12.37 ± 0.17	13.00 ± 0.12
Thr	3.33 ± 0.23	4.1 ± 0.07	3.70 ± 0.08
Ser	5.64 ± 0.12	6.4 ± 0.07	6.17 ± 0.03
Glu	17.79 ± 0.31	21.67 ± 0.16	24.78 ± 0.53
Pro	5.12 ± 0.17	4.17 ± 0.36	5.38 ± 0.33
Gly	7.37 ± 0.17	7.82 ± 0.09	4.15 ± 0.06
Ala	6.96 ± 0.04	7.05 ± 0.08	3.48 ± 0.02
Cys	0.29 ± 0.05	0.19 ± 0.01	1.91 ± 0.06
Val	6.03 ± 0.03	4.11 ± 0.06	2.24 ± 0.01
Met	1.05 ± 0.02	1.9 ± 0.09	0.78 ± 0.04
Ile	5.98 ± 0.26	6.66 ± 0.18	2.47 ± 0.01
Leu	8.34 ± 0.34	6.01 ± 0.16	7.17 ± 0.19
Tyr	3.19 ± 0.21	2.09 ± 0.02	3.36 ± 0.07
Phe	4.66 ± 0.22	3.15 ± 0.17	5.38 ± 0.14
His	2.24 ± 0.05	2.48 ± 0.04	2.24 ± 0.01
Lys	5.75 ± 0.13	5.21 ± 0.31	6.17 ± 0.27
Arg	5.63 ± 0.04	4.64 ± 0.03	7.62 ± 0.28

TABLE 2 | Molecular mass distribution of PPs and SePPs.

Molecular weight (Da)	The content of PPs (%)	The content of SePPs (%)
>3000	4.64 ± 0.001	5.41 ± 0.001
1500-3000	7.66 ± 0.001	7.52 ± 0.001
1000-1500	10.94 ± 0.002	10.61 ± 0.001
500-1000	20.17 ± 0.003	22.99 ± 0.003
<500	56.58 ± 0.006	53.47 ± 0.005

ameliorative effect of SePPs on oxidative damage *in vivo* in the heat stress and exercise fatigue-induced heatstroke rats. As shown in **Figure 2**. Heatstroke significantly decreased ($p < 0.05$) the CAT, SOD, GSH-Px, and GSH content in the serum of the rats in the model group by 61, 46, 13, and 73%, respectively, while the MDA content was 170% higher than that in the control group, suggesting stronger oxidative stress in the rats after heatstroke. Compared with the model group, significant differences were found in the CAT, SOD, MDA, GSH, and GSH-Px levels in serum between the SePro and SePPs groups ($p < 0.05$), while no statistical difference was observed in the MDA content levels in the serum between the PPs and model groups ($p > 0.05$). Additionally, there were no statistical differences between the SOD activity and MDA content in the serum of the rats in the SeMet and model groups ($p > 0.05$), while the serum SOD, GSH-Px activities, and MDA content of the rats in the Na_2SeO_3 group were not statistically different from those in the model group ($p > 0.05$). Our results indicated that SePPs could improve oxidative damage due to heatstroke by increasing the antioxidant enzyme activity and inhibiting lipid peroxidation. Furthermore, its ability to increase antioxidant enzyme activity in the body was higher than that of SePro, PPs, SeMet, and Na_2SeO_3, while the degree of lipid peroxidation inhibition was not statistically different from that of SePro ($p > 0.05$).

Effect of Different Selenium Supplements on the Serum Liver Enzyme Levels in Heatstroke Rats

The ALT and AST levels in the serum of the rats were determined to assess the impact of heat stress and exercise fatigue on the liver. It has been shown in **Figure 3** that the liver enzyme levels in the serum of the rats in the model group were significantly higher ($p < 0.05$), which were 2.0 and 2.2 times higher than those in the control group. The data indicate the impaired liver function after heatstroke. The SePro, SePPs, PPs, and SeMet groups displayed significantly lower ($p < 0.05$) liver enzyme blood levels than the model group, while the serum ALT in the Na_2SeO_3 group did not differ considerably from the model group ($p > 0.05$). SePro, SePPs, and PPs were more effective in reducing the serum ALT levels than SeMet and Na_2SeO_3, while SePPs were the most effective in lowering serum AST among the other treatment groups. Overall, SePPs were superior to other supplements in preventing liver damage.

Effect of Different Selenium Supplements on the GSH and GSH-Px Content in the Liver Tissue of Heatstroke Rats

Selenium is a vital component of GSH-Px and is closely related to the GSH system. Therefore, the GSH and GSH-Px content in the liver tissues were determined further. The results are shown in **Figure 4**. The GSH and GSH-Px content in rat livers was considerably reduced ($p < 0.05$) after heatstroke, which was 64 and 59% of the control, respectively. Supplementation with SePro, SePPs, PPs, and SeMet before heat stress and exercise improved the decrease in GSH and GSH-Px content ($p < 0.05$). However, Na_2SeO_3 supplementation did not significantly augment the GSH and GSH-Px levels in the liver ($p > 0.05$). The GSH contents in the SePPs group were higher than in the control group.

Histopathological Analysis of the Liver

The results of histopathological analysis were presented in **Figure 5**. The overall structure of the liver tissue in the control group was normal, with a complete hepatocyte structure, and a clearly visible central vein, while the hepatic sinusoids were arranged radially along the central vein, as shown by the yellow arrows. The white arrows indicated the hepatic sinusoidal macrophages, and no obvious inflammatory cell infiltration was evident in the tissue. In contrast, the overall structure of the liver tissue in the model group was abnormal, with a loose hepatocyte structure, hepatocyte necrosis, and the disappearance of nucleus fixation and lysis, as shown by the blue arrows, while the black arrows indicated the presence of inflammatory cell infiltration in the tissue. The addition of SePro, SePPs, PPs, and SeMet improved liver tissue damage to varying degrees, while Na_2SeO_3 supplementation did not facilitate significant improvement. The overall structure of the liver tissue was mildly abnormal in the SePro, PPs, and SeMet groups, with visible inflammatory cell infiltration, as shown by the black arrows. Moreover, significantly dilated hepatic sinusoids were evident in the SePro and SeMet groups, as shown by the yellow arrows. Notably, the SePPs group

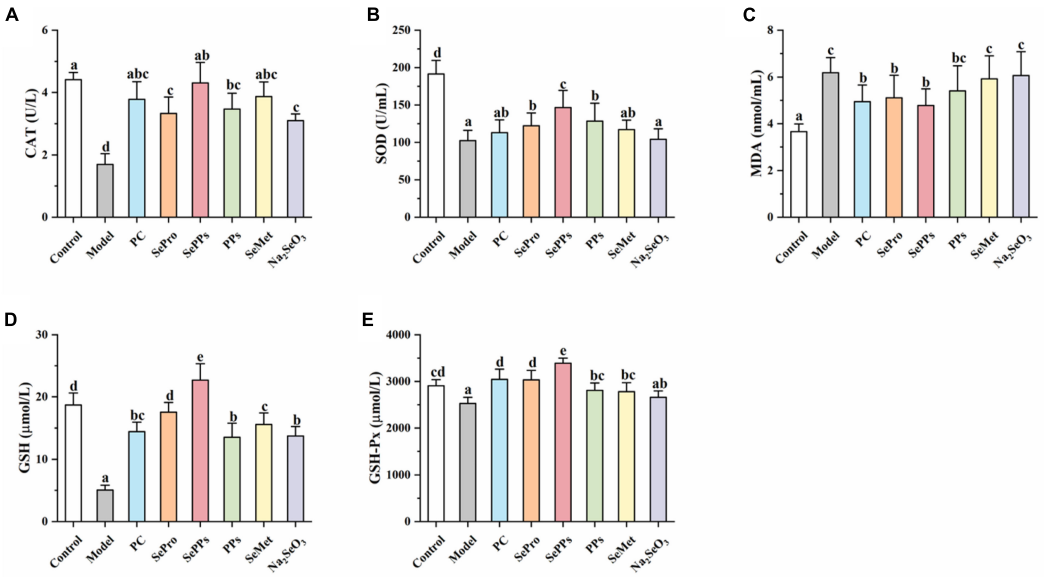

FIGURE 2 | Effects of different selenium supplements on CAT **(A)**, SOD **(B)**, MDA **(C)**, GSH **(D)**, and GSH-Px **(E)** in rat serum. Values are expressed as the mean ± SD, results marked with the same letters were not significantly different ($p > 0.05$).

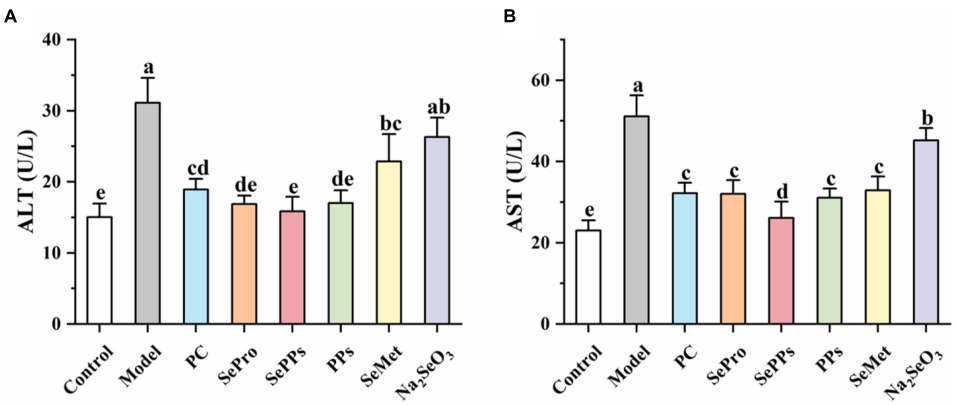

FIGURE 3 | Effects of different selenium supplements on ALT **(A)** and AST **(B)** in rat serum. Values are expressed as the mean ± SD, results marked with the same letters were not significantly different ($p > 0.05$).

displayed a complete hepatocyte structure, and a clearly visible central vein. The tissue did not show obvious sparing edema necrosis of the hepatocytes, and no noticeable inflammatory cell infiltration was apparent. Additionally, several new hepatocytes were visible, as shown by the green arrows in **Figure 5E**.

Effect of Different Selenium Supplements on the Inflammatory Factor Concentration in the Liver Tissue of Heatstroke Rats

Various physiological responses, such as the inflammatory response, occur *in vivo* in heat stress conditions. IL-1β, IL-6, and TNF-α are regarded as important indicators during heatstroke studies. As shown in **Figures 6A-C**, the IL-1β, IL-6, and TNF-α levels in the livers of the rats in the model group were 1.5, 1.5, and 1.4 times of in the control group ($p < 0.05$) after exposure to heat stress and exercise, indicating a stronger inflammatory response in the liver tissue. Compared to the model group, IL-1β, IL-6, and TNF-α were substantially lower in the SePro, SePPs, PPs, SeMet, and Na_2SeO_3 groups ($p < 0.05$). It seemed that selenium and nutritional protein supplementation suppressed these substances after heat stress and exercise, while SePro, SePPs, and PPs were more effective than SeMet and Na_2SeO_3.

Effect of Different Selenium Supplements on the Expression of Related Proteins in the NF-κB/IκB Signaling Pathway of Heatstroke Rat Liver Tissues

The NF-κB p65 and p-IκB protein levels in the different treatment groups were measured to investigate the role of the NF-κB/IκB

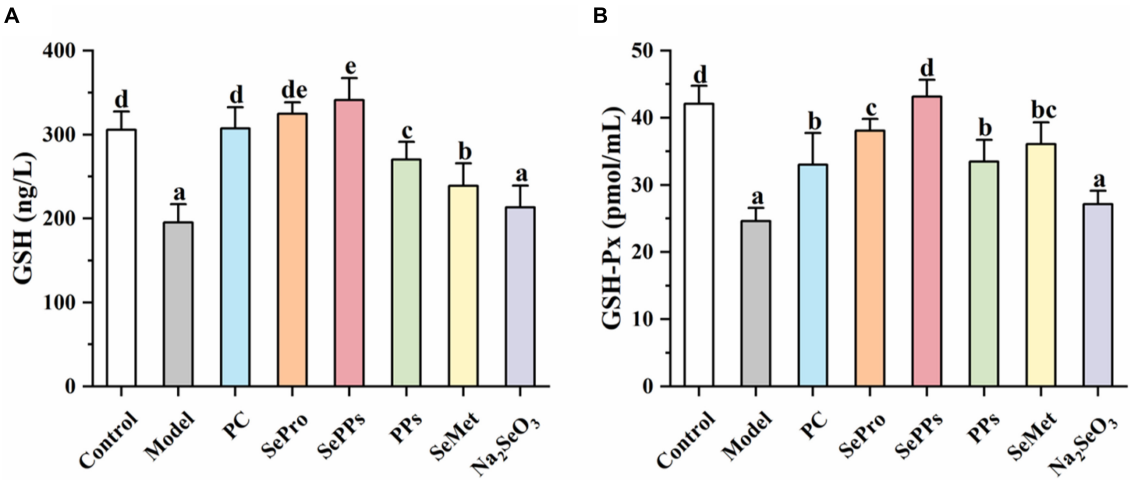

FIGURE 4 | Effects of different selenium supplements on GSH **(A)** and GSH-Px **(B)** in rat liver. Values are expressed as the mean ± SD, results marked with the same letters were not significantly different ($p > 0.05$).

signaling pathway in heatstroke. As shown in **Figures 6D-F**, the protein expressions of p-IκB and NF-κB p65 were upregulated in the livers of rats after heat stress and exercise. The SePPs treatments significantly reduced p-IκB and NF-κB p65 protein expressions compared with the model group ($p < 0.05$). The protein expression of p-IκB in SePro, PPs, SeMet, and Na_2SeO_3 treatment groups were not significantly different from that of the model group ($p > 0.05$). The results indicated that supplementation with selenium and nutritional proteins could improve the inflammatory response by inhibiting the expressions of p-IκB and NF-κB p65 protein *in vivo*. SePPs were the most effective of the selenium supplements, variation was evident in the regulation of different transcription factors, while the regulation of p65 protein expression was superior to that of p-Iκ B.

DISCUSSION

The morbidity and mortality of sports diseases have attracted increasing attention in recent years. However, in the context of dramatic global warming, there is no specific and effective treatment strategy for heat stress and exercise fatigue-induced heatstroke. Therefore, it is essential to study on the prevention of heatstroke. The primary aim of this study was to investigate the protective effect and potential action mechanism of SePPs in heat stress and exercise fatigue-induced heatstroke rats. This study focuses on the liver since it is more sensitive to heat stress, while liver injury and dysfunction represent the most common and often fatal pathological changes in almost all cases of heat stress (16). Additionally, the liver is the central organ for selenium metabolism. Selenium ingested *via* food is absorbed in the intestine and transported to the liver for metabolism, from where it is transported and distributed to other tissues in the body (17). This study selected honeysuckle as a PC due to its antioxidant and anti-inflammatory effect. Using Freund's adjuvant-induced arthritis rat model, Wu et al. found that honeysuckle extract inhibited the production of pro-inflammatory factors (TNF-α, IL-1β, IL-6, NO/iNOS, and cyclooxygenase-2 [COX-2]) and increased the activity of antioxidant enzymes (SOD, GSH-Px, and heme oxygenase-1 [HO-1]), attenuating arthritis symptoms (18). Honeysuckle also exhibits hepatoprotective effects (19).

Heatstroke causes oxidative damage, which is related to the pathophysiology of various disorders (20), including respiratory muscle dysfunction in heart failure (21) and skeletal muscle exhaustion *in vitro* (22). CAT, SOD, and GSH-Px are key components of the antioxidant enzyme system in living organisms and play a significant role in maintaining oxidative and antioxidant equilibrium in the body. GSH helps maintain normal immune system function, displays antioxidant effects, and integrates detoxification. MDA is the end product of lipid peroxidation and induces the cross-linking polymerization of proteins, nucleic acids, and other biological macromolecules. This study showed a decrease in antioxidant enzyme activity and an increase in lipid peroxidation after heat stress and exercise, consequently causing oxidative stress. Zhang et al. found that selenium-containing soybean antioxidant peptides inhibited D-galactose-induced liver injury and oxidative brain damage *via* the MAPK/NF-κB pathway, and played a key role in reducing organic oxidative damage during inflammation and aging. They compared the antioxidant activity of inorganic selenium, organic selenium, and selenium-free peptides and found that the effect of the selenium-enriched peptide treatment group was more significant (23). Consistent with these findings, this study revealed that PPs exhibited an ameliorative effect on the oxidative damage caused by heat stress and exercise fatigue. However, the effect of the SePPs treatment group was superior to PPs and other forms of selenium supplementation, indicating that both selenium and peptides play a vital role in antioxidant activity. Many studies have reported the antioxidant effect of selenium (24), while its bioavailability is essential for the strength of functional expression. We improved selenium

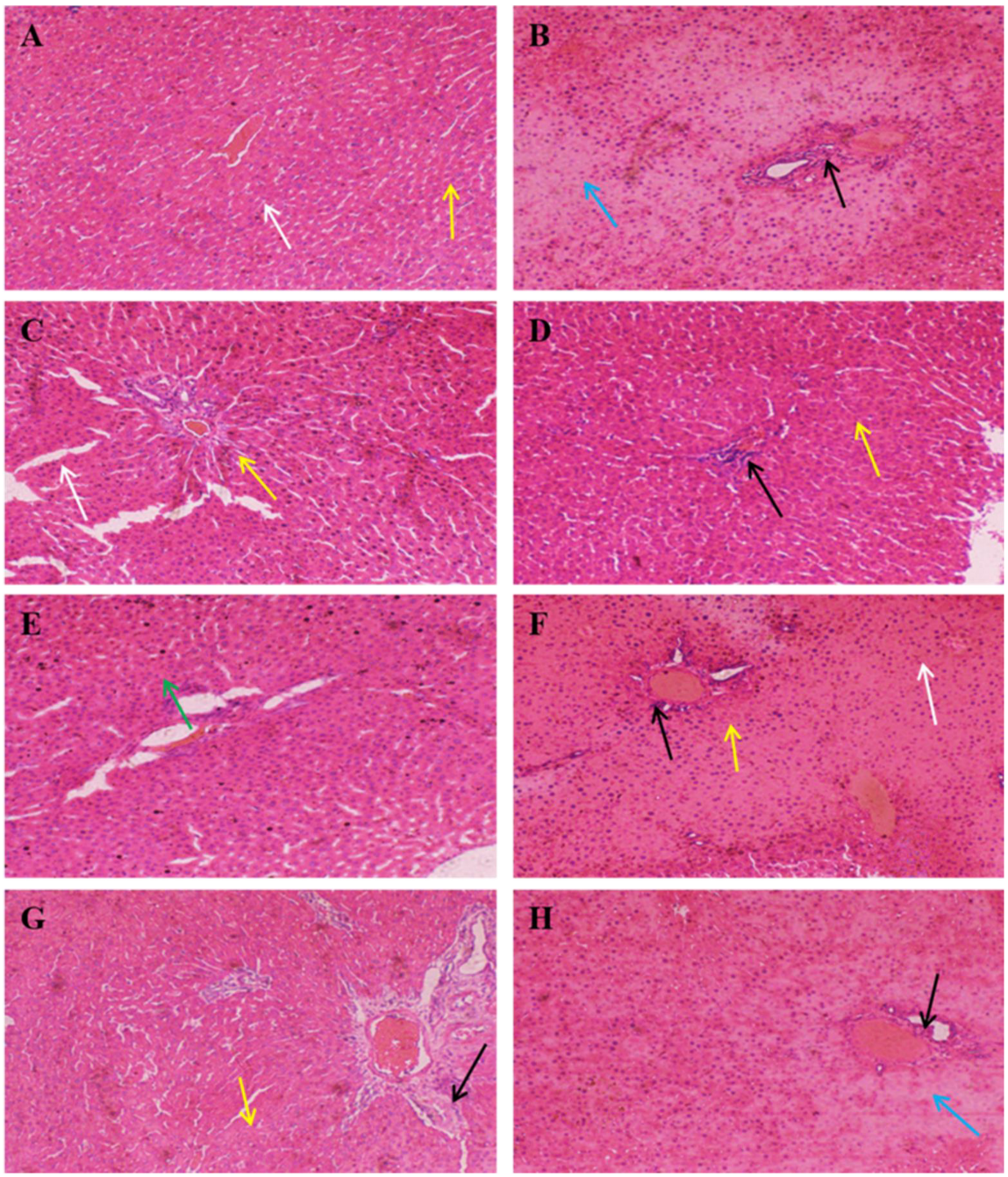

FIGURE 5 | Histopathology of liver (H&E, × 100). Yellow arrows indicate hepatic sinusoids; blue arrows indicate necrosis of hepatocytes and disappearance of nuclei consolidation and lysis; white arrows indicate hepatic sinusoidal macrophages; black arrows indicate inflammatory cells; green arrows indicate neoplastic cells. **(A)** Control group, **(B)** Model group, **(C)** PC group, **(D)** SePro group, **(E)** SePPs group, **(F)** PPs group, **(G)** SeMet group, **(H)** Na_2SeO_3 group.

bioavailability *via* selenium biofortification with soy protein, soy peptides, and methionine as carriers, respectively. Protein stability and transport pathways *in vivo* determine the true efficacy of functional applications. The oral bioavailability of such macromolecules is generally low due to poor membrane permeability. Proteins are typically denatured by pepsin and produce a mixture of peptides with molecular weights > 10 kDa. These gastric digestion products are further hydrolyzed by pancreatic enzymes, indicating that they exhibit low stability in the gastrointestinal tract and are susceptible to degradation by multiple digestive enzymes. Therefore, they are difficult to be absorbed by the human body completely (25). SeMet is absorbed through the intestinal methionine transporter after ingestion, following the active amino acid absorption pathway.

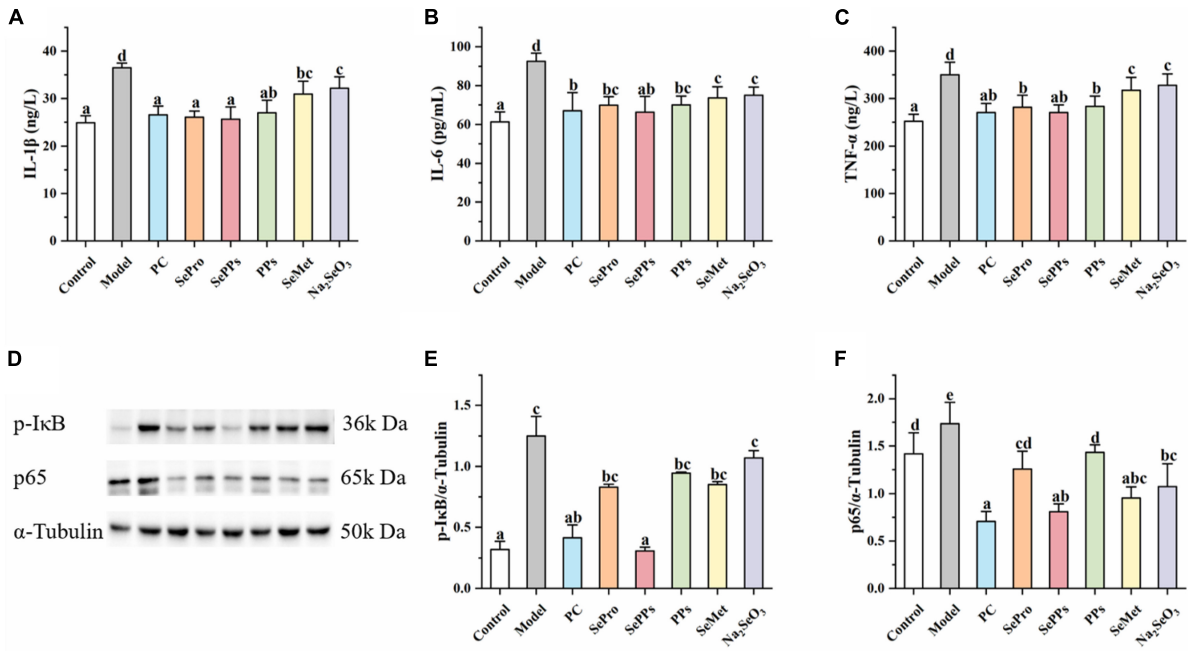

FIGURE 6 | Effects of different selenium supplements on IL-1β **(A)**, IL-6 **(B)**, TNF-α **(C)**, Western blot analysis **(D)**, p-IκB **(E)**, and p65 **(F)** in rat liver. Values are expressed as the mean ± SD, results marked with the same letters were not significantly different ($p > 0.05$).

SeMet and Met can share the same Na^+-dependent, carrier-mediated transport mechanism (26), which is also an important way for multiple amino acids to be absorbed by the intestine. This may decrease SeMet absorption efficiency due to the structural similarity of individual amino acids and mutual competition. The higher bioavailability exhibited by SePPs may be due to their higher stability and membrane permeability, as well as their rapid *in vivo* transport and less saturable carriers. Furthermore, the results indicated that different forms of selenium exhibit varying bioavailability, while that of organic selenium was significantly higher than inorganic selenium. The body can absorb inorganic and organic selenium *via* the digestive tract, particularly the small intestine. However, a large amount of inorganic selenium is lost *via* urine as selenite and selenate before further metabolism, resulting in the bioavailability of selenium as selenite and selenate at levels lower than organic selenium (27). In line with previous findings, acute heat exposure increased lipid peroxidation and protein oxidation in rat plasma, ultimately resulting in oxidative damage to the body (28). As a metabolic crossroads, the liver is vulnerable to heat stress and exercise state, disturbing the metabolic equilibrium of the liver (29). Moreover, the liver is high in selenium, accounting for 29% of the total selenium in rats (27). It is the first organ encountered after selenium absorption in the small intestine and plays a crucial role in selenium metabolism (30). Consequently, more research regarding heat stress and exercise fatigue-induced liver injury was conducted.

The liver secretes several endogenous and exogenous compounds during detoxification. ALT and AST are hepatic enzymes, the levels of which can be used as key indicators of liver function. Our results showed that heatstroke compromised the integrity of hepatocyte membranes, allowing aminotransferases to escape into the serum from the cytoplasm. Generally, the increase in the degree of ALT and AST in serum reflects the degree of hepatocyte injury. ALT is mainly present in the cytoplasm of hepatocytes, while AST is primarily distributed in the cytoplasm and mitochondria of hepatocytes. Elevated ALT or AST level in the blood suggested hepatocyte membrane injury and high membrane permeability or organelle damage, respectively (31). Guo et al. found that selenium biofortified maize peptide exerted a substantial ameliorative impact on the high ALT and AST caused by concanavalin A (32). Similarly, the present study revealed that advanced supplementation with different forms of selenium reduced liver damages induced by heat stress and exercise fatigue and PPs displayed hepatoprotective benefits.

As protein degradation products, peptides play an essential role in the development of various liver disorders. In the investigation of Lin et al., marine collagen peptides inhibited early alcoholic liver injury in female rats by improving oxidative stress and lipid metabolism (33). Lv et al. observed that maize peptides significantly reduced MDA, NO, hydroxyproline (HYP), transforming growth factor-β1 (TGF-β1) levels and lactate dehydrogenase activity in the liver and substantially increased the SOD level. The action mechanism of maize peptides might be related to their antioxidant activity in hepatocytes and TGF-β1 secretion inhibition (34). Recently, SePPs were found to exhibit better immunomodulatory effects than PPs in a mouse model involving cyclophosphamide-induced immunosuppression (13). In our study, SePPs had a more significant impact on the GSH system than PPs. The serum indicator results demonstrated

that SePPs were most effective in regulating GSH and GSH-Px. Consequently, the GSH and GSH-Px levels in the liver were investigated further, showing that the GSH system was altered after heat stress and exercise fatigue. Using a broiler model, Yang et al. found that acute heat exposure could inhibit the mitochondrial respiratory chain activity, resulting in excessive ROS production and eventually oxidative stress (35). Excessive ROS production overwhelms cellular antioxidant defenses, causing damage to various cellular components (36). The liver is responsible for neutralizing excess ROS by disrupting the mitochondrial membrane proton gradient, causing oxidative damage by breaking the loops of enzymes, cellular lipids, and mitochondrial membranes in the liver. Furthermore, ROS can also act as signaling molecules, affecting the expression of related genes, requiring regulation by the antioxidant system (37–39), which includes GSH and GSH-Px. Similar to the results obtained by Liu et al. (12), the GSH content and GSH-Px activity in the serum and liver of the rats in the SePPs group were higher than those in both PPs group and control group, which may be attributed to the unique relationship between selenium and the GSH system. The increased SePPs on GSH-Px activity may be related to the presence of SeCys peptides in SePPs. Since SeCys represents the active GSH-Px center (40), SePPs can be used as a synthetic GSH-Px and increase its activity, converting more GSH to oxidized GSH (GSSG). Then, catalyzed by GSH reductase, GSSG can be reduced to GSH again by nicotinamide adenine dinucleotide phosphate (NADPH) (41). Throughout the redox cycle, higher GSH-Px activity stimulated the involvement of GSH in the reaction, resulting in higher GSH levels in the SePPs-treated group. This indicated that SePPs more successfully regulated the GSH system in the liver than PPs, reducing oxidative damage. In addition, the GSH content affected the TNF-α sensitivity to hepatocyte destruction. The cell death process includes the mitochondrial release of cytochrome C, while a decrease in the cytoplasmic GSH content is necessary for cytochrome C to promote apoptosis (42). According to Matsumaru et al., GSH depletion in the body enhanced TNF-α sensitivity to induce apoptosis in primary murine hepatocytes (43). The histological results of the current research showed higher hepatocyte death after heatstroke, which was alleviated to varying degrees by SePro, SePPs, PPs, and SeMet supplementation. This may be related to GSH content changes in the liver.

The inflammatory response is another significant mechanism of heat stress and exercise fatigue-induced heatstroke (44). Selenium has been demonstrated to be able to attenuate the inflammatory response (45). In this study, SePPs inhibited the release of inflammatory factors (IL-1β, IL-6, and TNF-α) in the liver. The p-IκB and p65 protein expressions in the NF-κB/IκB signaling pathway were measured in the liver tissue to investigate the action mechanism of SePPs in suppressing the inflammatory response (see **Figure 7**). NF-κB is a nuclear transcription factor that also functions as an inflammatory mediator. The p50/p65 heterodimer is the common form of NF-κB present in hepatocytes and is inactivated by the binding of IκB to subunit p50/p65 in the cytoplasm (46). Xie et al. found that the NF-κB/IκB pathway plays a vital role in heat stress-induced ROS generation and cytotoxicity in the microvascular endothelial cells in rat lungs (47). In accordance to their results, heatstroke affected the NF-κB/IκB pathway and produced phosphorylated IκB, which was released from subunit p50/p65. It was ubiquitinated, converting subunit

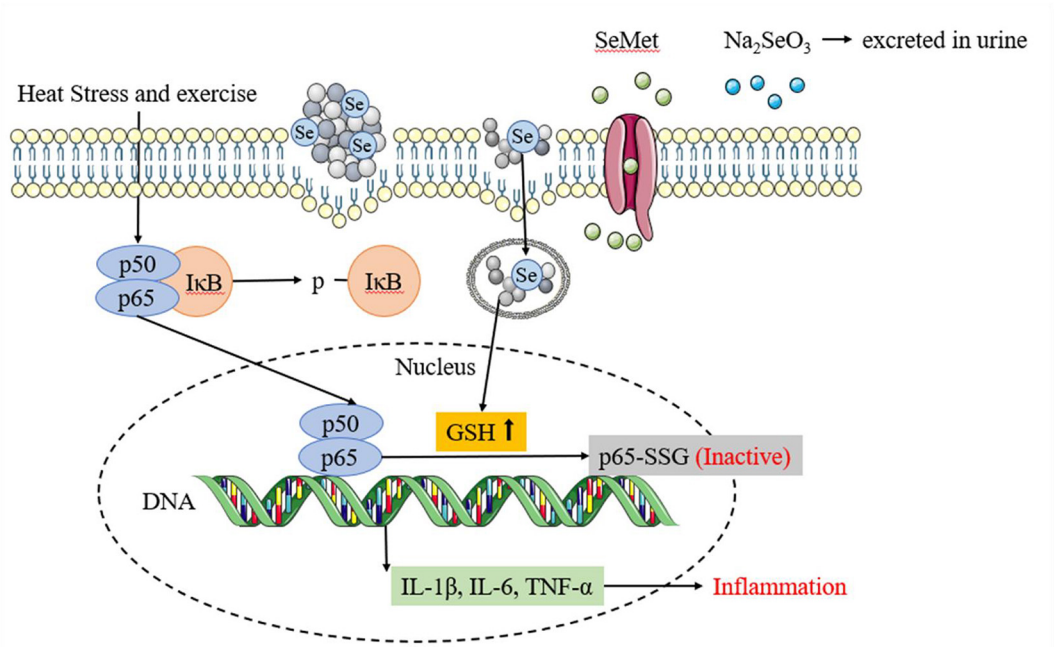

FIGURE 7 | Different selenium supplements inhibits inflammatory response by affecting NF-κB/IκB signaling pathway.

p50/p65 from an inhibited to an activated state, while activating the transcription of the gene-encoding proteins involved in the cellular response to stress factors (48). NF-κB controls pro-inflammatory cytokines and chemokines by increasing cell proliferation and stimulating angiogenesis. Consequently, limiting NF-κB activation potentially blocks cytokine expression, preventing the inflammatory response. Ge et al. reported that selenium supplementation attenuated Cd accumulation in the heart and reduced cardiac injury by affecting the NF-κB/IκB signaling pathway (49). In this investigation, supplementation with different forms of selenium and PPs before heat stress and exercise inhibited the p-IκB and p65 protein expression. Comparative analysis of the protective effects showed that SePPs inhibited the inflammatory response more effectively than other selenium supplements, indicating that selenium exerts an anti-inflammatory impact, while selenium and peptide binding is more easily absorbed and utilized by the body. SePPs could reduce inflammation by inhibiting the expressions of p-IκB and p65 proteins in the NF-κB signaling pathway. The DNA-binding activity of NF-κB can be regulated by redox, while the oxidation of its cysteine residues is necessary for the initiation of downstream effector proteins (50). The selenium and GSH Sytems display a unique relationship, rendering the liver GSH and GSH-Px significantly higher in the SePPs group than in the other groups. GSH can be engaged in various cellular reactions and can directly or indirectly modify the redox state of the liver *via* enzymatic reactions to scavenge free radicals and other reactive oxygen species (51). Furthermore, GSH also functions as a modifier of redox-sensitive proteins, especially since the protein contains cysteine and has a low pKa value. GSH can form mixed disulfides with cysteine in target proteins, a process known as glutathionylation (52). It is considered as a redox-dependent modification with potential relevance to signal transduction, metabolism, inflammation, and apoptosis, suggesting that GSH is an essential antioxidant and a crucial signaling molecule. Zhou et al. showed that GSH supplementation inhibited FKB-induced hepatocyte death *via* NF-κB and MAPK signaling (53). Proteomic studies have identified many proteins regulated by glutathionylation, including actin, Protein Kinase (PK) A, PKC, and NF-κB (54). NF-κB includes various subunits, of which p50 and p65 are the most abundant. The cysteine residue in the DNA-binding domain of the NF-κB p50 subunit can be glutathione, resulting in activity loss (55). The p65 subunit can also be modified by glutathionylation, causing a loss in activity (56). Since only p65 NF-κB is responsible for transcriptional activation (57), the effect of glutathionylation of the p65 subunit should have a stronger impact on NF-κB activation than that of the p50 subunit. These findings suggest that internal cysteine glutathionylation may be an important mechanism for protein expression regulation. Therefore, the reduced p65 protein level may also be due to the elevated GSH level leading to p65 glutathionylation. This protects sensitive cysteine residues from oxidation, restores protein function in oxidative stress conditions, and inhibits NF-κB activation, consequently reducing the degree of inflammation.

CONCLUSION

This study provides *in vivo* evidence that SePPs protect rats from heat stress and exercise fatigue-induced heat stroke. SePPs improve the antioxidant capacity and inflammatory response of the liver in rats caused by heatstroke and reduce liver damage by inhibiting p-IκB and p65 protein expressions in the NF-κB/IκB pathway. It suggests that NF-κB/IκB may serve as a target for heatstroke treatment. In terms of boosting the GSH system, SePPs have a more significant impact than PPs. A comparison between the different selenium supplements revealed that combining selenium and peptides was more successful *in vivo*, suggesting that SePPs can be used as a preferential selenium resource. Alternatively, our study illustrates the potential of SePPs to prevent heatstroke and mitigate sports injury. Future research can focus on the structural interactions and metabolic pathways of SePPs to thoroughly understand their mode of action.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Pony Testing International Group Co., Ltd. (PONY-2021-FL-15).

AUTHOR CONTRIBUTIONS

XC designed the research. XC and WL carried out experiments. XC and JZ analyzed the data. XC and HL wrote the initial manuscript, with contributions from YX and XL. All authors contributed to the article and approved the submitted version.

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Enhancing organic selenium content and antioxidant activities of soy sauce using nano-selenium during soybean soaking

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Nano-selenium has a greater potential than inorganic selenium in preventing selenium-deficiency diseases due to its higher safety. In this study, spherical nano-selenium particles (53.8 nm) were prepared using sodium selenite, ascorbic acid and chitosan. Selenium-enriched soy sauces were prepared by soaking soybean in nano-selenium and sodium selenite solutions (2–10 mg/L), respectively. Total selenium and organic selenium contents of soy sauces prepared by nano-selenium and sodium selenite were increased by 32–191-fold and 29–173-fold compared to the control (without selenium), and organic selenium accounted for over 90% of total selenium. Soy sauce prepared using 6 mg/L nano-selenium had the strongest antioxidant activities, which were 9.25–28.02% higher than the control. Nano-selenium (6 mg/L) markedly enhanced the koji's enzyme activities (9.76–33.59%), then the latter promoted the release of total phenolics (27.54%), total flavonoids (27.27%) and the formation of free amino acids (16.19%), Maillard reaction products (24.50%), finally the antioxidant activities of selenium-enriched soy sauce were enhanced.

KEYWORDS

nano-selenium, soy sauce, antioxidant activity, *Aspergillus oryzae*, active ingredient

Highlights

- Organic selenium content of soy sauce was enhanced by nano-selenium during soybean soaking.
- Antioxidant activities of soy sauce were enhanced by nano-selenium during soybean soaking.
- Nano-selenium promoted microorganism growth and enzymes secretion during soy sauce making.
- Soybean soaking and microorganism converted nano-selenium into organic selenium.
- Enzymes from *A. oryzae* promoted formation and release of antioxidant compounds in soy sauce.

Introduction

Selenium (Se), a micronutrient with antioxidant properties, is essential for all life forms including human beings. It often exists in membrane selenoproteins (including deiodinases, etc.) and free selenoproteins (methionine sulfur oxide reductase, glutathione peroxidase, thioxycyclin reductase, etc.) in the form of coenzyme factors or cogroups (1, 2). These selenoproteins play an important role in scavenging metabolic reactive oxygen species (ROS) in human body, which are closely related to the formation of aging, cancer, Alzheimer's disease, cardiovascular diseases, disorder in human reproduction, etc. (1, 3). Unfortunately, billions of people in the world can not intake the recommended minimum dose of selenium (60 μg/adult/day) due to the uneven distribution of selenium in land. To make matters worse, the selenium intake of approximately 1/3 people in China is less than 25 μg/adult/day (4, 5). Therefore, it is of great significance to develop commercially available and affordable selenium-enriched foods and prevent the prevailing selenium-deficiency of people in the world.

Soy sauce is a necessary fermented condiment in China, Japan, Korean and other Southeast Asia Countries. Nowadays, over 70% of soy sauce is produced using soybean, wheat flour and salt as materials according to high-salt liquid-state fermentation technology in China (1). Because *Aspergillus oryzae* 3.042 is a "generally recognized as safe" strain and secrets plentiful enzymes suitable for the production of fermented foods, which is widely used in the manufacturing of soy sauce, soybean paste, *Sufu*, *Douchi*, etc. (6). The main manufacturing processes of soy sauce include soybean washing, soaking, steaming, cooling, inoculating (*Aspergillus oryzae*), koji preparation, moromi preparation (22% saline:koji = 2.2:1, w/v), fermentation (6 months), etc. (7, 8). Soy sauce contains abundant small molecular peptides, free amino acids (FAAs), saccharides and Maillard reaction compounds, and it has been proved to possess a strong antioxidant activity (3, 7, 9). The cysteine, methionine and small molecular peptides are the potential carriers of selenium to form organic selenomethionine (SeMet) and selenocysteine (SeCys) *via* microbial transformation during soy sauce fermentation (10–12). Organic selenium has far higher safety and bioavailability for human compared to inorganic selenium (3, 4). Furthermore, the strong antioxidant activity of soy sauce is conducive to reduce the toxic high valence selenium to non-toxic low valence selenium or keep the stability of low valence selenium. Thus, soy sauce is an ideal carrier of selenium supplement for human. However, it is not acceptable to add inorganic selenium (Na_2SeO_3 and Na_2SeO_4) during soy sauce production due to its strong toxicity and the possible residual of inorganic selenium. Thus, it is prerequisite to enhance the selenium content in soy sauce by applying safe selenium.

Gao et al. (1) developed a selenium-enriched soy sauce by replacing soybean with selenium-enriched soybean. Results indicated that the contents of organic selenium and total selenium reached 59.6 and 79.3 μg/kg in the selenium-enriched soy sauce, respectively, which were 10.6 times and 11.1 times more than those in the ordinary soy sauce. Unfortunately, the selenium content is still difficult to meet the minimum recommended intake for human beings (50 μg/day), because the daily per capita consumption of soy sauce is approximately 10 mL in China and the other Asia countries. Furthermore, the high price of soy sauce produced by selenium-enriched soybean is not accepted by most consumers. The preparation and application of nano-selenium in foods have drawn more and more attention because of the low toxicity and high safety (13, 14). Furthermore, the antioxidant activity and immunoregulation ability of nano-selenium *in vivo* and *in vitro* are also proved by various researchers (2, 14, 15). So far, selenium-enriched soy sauce produced by nano-selenium has not been reported.

Hence, the aims of this research were to (i) develop a nano-selenium solution and selenium-enriched soy sauce using the prepared nano-selenium solution, (ii) evaluate the effect of nano-selenium on the antioxidant activities of soy sauce, (iii) clarify the enhancement mechanism of antioxidant activities of seleniums-enriched soy sauce.

Materials and methods

Strain and chemicals

A. oryzae 3.042 was supplied by Guangdong Institute of Microbiology (Guangzhou, China). Soybean, wheat flour and sodium chloride (NaCl) were purchased from a local supermarket (Zhenjiang, China). Na_2SeO_3, ferrozine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Cicalteu phenol reagent, 6-hydroxy-2,5,7,8-tetramethylchro-man-2-carboxylic acid (Trolox), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), gallic acid and rutin were obtained from Sigma (St. Louis, MO, USA). The other chemicals utilized in this research were ordered from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Schematic overview of the experimental program

The experimental program of this work was shown in Figure 1, the detailed experimental methods were described in the following sections.

Preparation of nano-selenium

Ten milliliters of 0.1 mol/L Na_2SeO_3 and 12 mL of 10 g/L chitosan solution (95% deacetylation degree) were

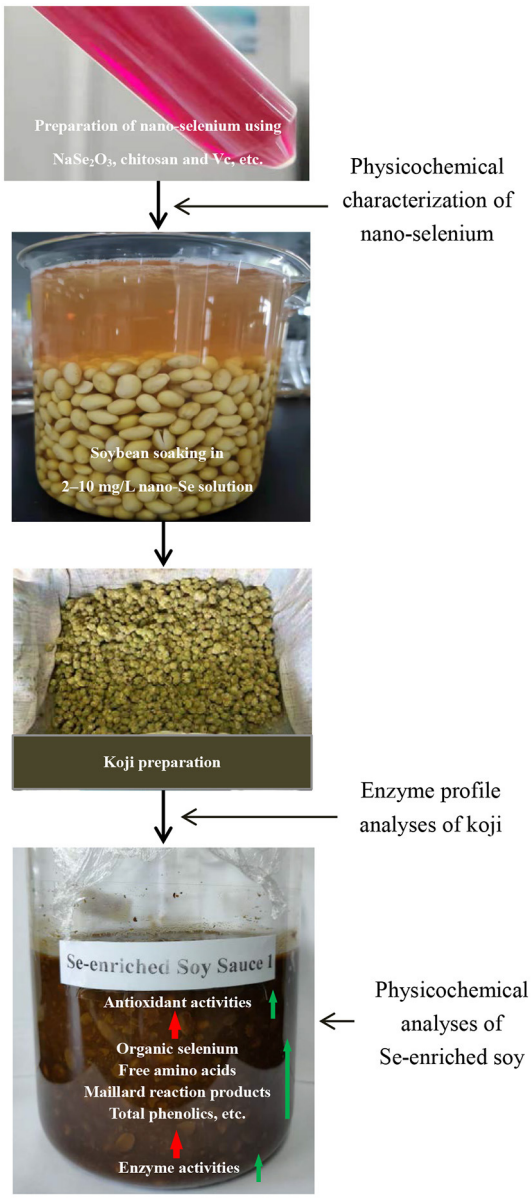

FIGURE 1
Schematic overview of the experimental program.

placed in a 100-mL brown conical flask with a stopper. After shaking (100 r/min) in a constant temperature shaker (Changzhou Jintan Jingda Instrument Manufacturing Co., LTD, Changzhou, China) at 30 °C for 30 min, 10 mL of 0.5 mol/L ascorbic acid solution was added and distilled water was added until the total volume was 40 mL. After the mixture was evenly mixed, 0.025 mol/L (measured by selenium) red nano-selenium solution was obtained by shaking in the constant temperature shaker for 1 h at 30 °C. The resulting nano-selenium solution was kept at room temperature in the dark for further use.

Physicochemical characterization of nano-selenium

The morphology and composition of the prepared nano-selenium were investigated by X-ray energy dispersive spectrometer (EDS) and transmission electron microscope (TEM).

Preparation of soy sauce

Soy sauce was prepared in light of the report of Gao et al. (7) with minor modifications. Firstly, soybean was washed thrice with tap water. The soybean were soaked in tap water, ascorbic acid solution (40 μg/mL), chitosan solution (9 μg/mL), ascorbic acid solution (40 μg/mL) + chitosan solution (9 μg/mL), sodium selenite solution (2–10 mg/L, calculated by selenium) and nano-selenium solution (2–10 mg/L, calculated by selenium) at room temperature for 12 h, respectively, soy sauces produced using the above soybean were named as the control, AA, CTS, AA+CTS, SS2–10 and NS2–10. Secondly, the soaked soybeans were steamed, cooled, inoculated with *A. oryzae* 3.042 and mixed with wheat flour, separately, then the mixtures were cultured in incubators at 30 °C until the mixtures were covered by yellowish to green spores (mature koji) (8). The activities of α-amylase, glucoamylase, cellulase, acid protease, neutral protease, and pectinase of kojis were determined based on the methods reported by Gao et al. (8) and Meini et al. (16). Thirdly, the kojis were thoroughly blended with 3-fold of saline (18%, w/v) to prepare moromis in 2-L beakers, and then the beakers were sealed with plastic membrane and incubated in a 35 °C incubator. *L. plantarum* with a final concentration of 1×10^6 cells/mL moromi was inoculated into all moromis on the first day. *Z. rouxii* with a final concentration of 2×10^6 cells/mL moromi (pH < 5.5) was introduced into all moromis on the 5th day. Finally, all soy sauces were taken on 180 d and kept at 4 °C refrigerator for analyses.

Determination of total selenium and organic selenium contents

Atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS) were utilized to determine the inorganic selenium and total selenium contents of soy sauces based on the report of Zhao et al. (17). Organic selenium content was obtained by subtracting inorganic selenium content from total selenium content.

Determination of antioxidant activities

DPPH radical scavenging activity

In comparison to the positive control (ascorbic acid), the DPPH radical scavenging activity of soy sauces was determined in light of the reported method (18). The percentage of DPPH radical scavenging activity of soy sauce was calculated using the following equation:

$$\text{DPPH radical scavenging efficiency } (\%) = \frac{A_0 - (A_1 - A_s)}{A_0} \times 100$$

Where A_0 is the absorbance of blank solution containing only DPPH, A_1 stands for the positive control with DPPH solution or soy sauce with DPPH, and A_s means the positive control without DPPH solution or soy sauce solution without DPPH. The results were described as ascorbic acid equivalent antioxidant capacity (μg AAE/mL soy sauce), which was designated as the quantity of ascorbic acid (in μg) with the equal antioxidant activity to 1 mL of soy sauce.

ABTS radical scavenging activity

Compared to the positive control (Trolox), the ABTS radical scavenging capacity of soy sauces was assessed based on the protocol reported by Lee et al. (19). The following equation was used to determine the ABTS scavenging efficiency:

$$\text{ABTS radical scavenging efficiency } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

Where A_c is the absorbance of control ($ABTS^{\bullet+}$ solution in the absence of the positive control or soy sauce) and A_s is the absorbance of sample ($ABTS^{\bullet+}$ solution with the positive control or soy sauce). The resulting data were described as Trolox equivalent antioxidant capacity (TEAC, μmol TE/mL soy sauce), which was designated as the quantity of Trolox (in μmol) with the equal antioxidant activity to 1 mL of soy sauce.

Evaluation of reducing power

The reducing power of soy sauces was determined with a spectrophotometer according to the reported method with some modifications (20). The reducing power was presented with μg ascorbic acid equivalent (μg AAE)/mL soy sauce.

Evaluation of metal ion chelating activity

The chelating activity of soy sauces was evaluated in light of the reported approach with few modifications (21). The assay was carried out by adding EDTA solution or distilled water to replace soy sauce for the positive (Ethylenediaminetetraacetic acid, EDTA) and blank control. The following equation was used to calculate the Fe^{2+} chelating activity:

$$\text{Metal ion chelating efficiency } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

Where $A_c$is the absorbance of the control (distilled water), and A_s is the absorbance of sample (soy sauce or EDTA). In this assay, EDTA was utilized as the standard antioxidant, and data were exhibited as μg EDTA equivalent (μg EE)/mL soy sauce.

Analyses of proximate indices

Total sugar and reducing sugar contents of soy sauces were determined in light of AOAC methods 992.23, 925.35 and 923.09, respectively (22). Total titratable acidity and formaldehyde nitrogen were assessed with titration methods (23). Total solids content of soy sauces was evaluated with AOAC method 990.2 (24). A volumetric titration with $AgNO_3$ was used to determine the salt content (expressed as NaCl, g/L) of soy sauces based on Mohr's method (25). While non-salt soluble solids content (g/L) of soy sauces was obtained by subtracting salt content from the total solids content (105 °C until constant weight).

Evaluation of total phenolics and total flavonoids contents

Total phenolics content of soy sauces, which was expressed as milligram gallic acid equivalents (GAE) per milliliter of soy sauce (mg GAE/mL soy sauce), was assessed using the Folin-Ciocalteu method according to the literature with minor revision (26). The total flavonoids content in soy sauce, which was expressed as milligram rutin equivalents per milligram of soy sauce (mg RE/mL soy sauce), was evaluated with a colorimetric method using rutin as the standard.

Determination of Maillard reaction products

After a 15-fold dilution of soy sauce with distilled water, it was filtrated with micropore films (0.45 μm of pore size) before further analysis. The Maillard reaction products in soy sauce were determined using the absorbance of diluted soy sauce at 420 nm multiplying dilution multiple.

Determination of free amino acids contents

The diluted soy sauce was filtrated with the micropore filter (0.45 μm pore size, Sangon Biotech, Shanghai, China) before FAA analysis, then the resulting filtrate was utilized to

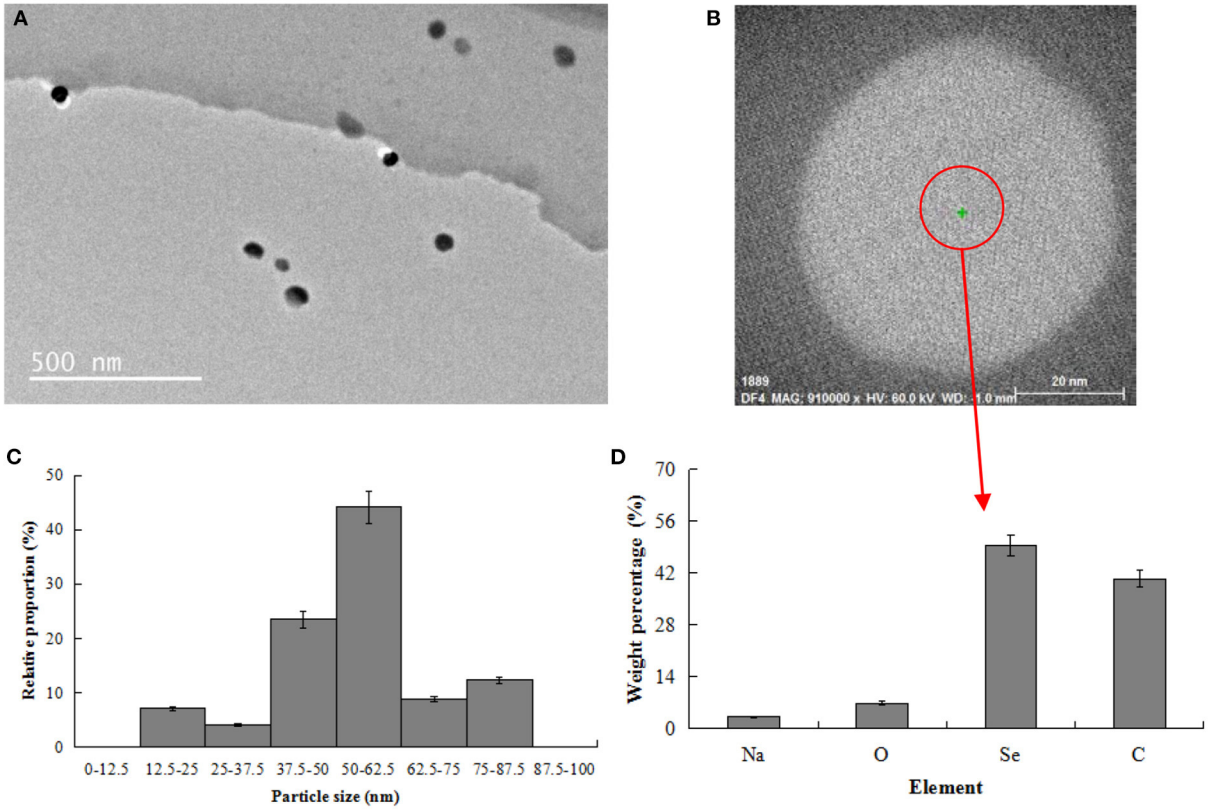

FIGURE 2
Physicochemical characterization of nano-selenium. **(A)** TEM image of nano-selenium (500 nm); **(B)** TEM image of nano-selenium (20 nm); **(C)** Particle size distribution of nano-selenium; **(D)** Element distribution of nano-selenium.

measure the FAA composition with the technique reported by Gao et al. (8) using a PICO·TAG amino acid analysis column (3.9 mm i.d. × 150 mm height; Waters Ltd., Milford, MA, USA) connected to an high-performance liquid chromatography (Waters Ltd., Milford, MA, USA). The detection was performed at the wavelength of 254 nm at 38 °C. The injection volume was 10 μL, and the flow rate of mobile phase was 1.0 mL/min. The standard amino acids (amino acid standard solution, type H, Sigma AAS18, St Louis, MO, USA) were used to determine the concentrations of amino acids in soy sauce.

Determination of antioxidant activities of ingredients in NS6

The antioxidant activities of soluble soybean polysaccharide (37,100 μg/mL), chitosan (9 μg/mL), ascorbic acid (40 μg/mL), sodium selenite (13.14 μg/mL), nano-selenium (6 μg/mL) and free amino acids (47,590 μg/mL) in NS6 were measured to clarify the contribution of nano-selenium to the enhanced antioxidant activities of selenium-enriched soy sauce.

Statistical analyses

All assays were carried out thrice, and all data were described as mean ± standard deviations. The obtained results were analyzed using one-way analysis of variance (ANOVA). The differences at p value below 0.05 were considered to be statistically significant unless otherwise specified. All calculations were performed by SPSS 15.0 (version 15.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Physicochemical characterization of nano-selenium

As shown in Figure 2A, the nano-selenium particles prepared in this work showed a relatively uniform dispersion state in the nano-selenium chitosan system. As shown in Figure 2B, the morphology of nano-selenium was complete and presented a regular spherical state. As shown in Figure 2C, the

diameter of most spherical nano-selenium ranged from 37.5 nm to 62.5 nm, with an average diameter of about 53.8 nm. Chen et al. (27) synthesized nano-selenium particles with an average diameter of 43.2 nm and high uniformity using chitosan as template, suggesting that the nano-selenium particles prepared in the present work were successful.

As shown in Figure 2D, the nano-selenium particles contained four main elements: Se, C, O and Na, they accounted for 49.38, 40.44, 6.93, and 3.25%, respectively. C and O were derived from soft template chitosan [$(C_6H_{11}NO_4)_n$] and reduced Na_2SeO_3, and Na and Se were derived from reduced Na_2SeO_3. N element was adjacent to C and O elements, and the content of N in nano-selenium was low, which was not easy to be identified by energy analysis spectrometer. Thus, N element was not detected in this experiment. N element in nano-selenium particles prepared using modified chitosan as template was not detected as well in a previous investigation conducted by Bai et al. (15), which contained 13.12% C, 11.56% O, 49.95% Cu, and 25.37% Se. Different ingredients used in the nano-particles and detection scopes of energy analysis spectrometer were attributed to the different element compositions in these two nano-particles.

Total selenium and organic selenium contents

The contents of total selenium (TSe), inorganic selenium (ISe) and organic selenium (OSe) in soy sauces increased with the enhancement of selenium concentrations, which were 182.74–1,079.15 μg/kg, 8.76–100.29 μg/kg and 173.98–978.86 μg/kg and significantly higher than those of the control, AA, CTS and AA+CTS ($F_{TSeSS2/control}$ = 2,717.243, $p_{TSeSS2/control}$ = 0.000, $F_{TSeSS4/control}$ = 2,400.458, $p_{TSeSS4/control}$ = 0.000, $F_{TSeSS6/control}$ = 1,165.772, $p_{TSeSS6/control}$ = 0.000, $F_{TSeSS10/control}$ = 795.401, $p_{TSeSS10/control}$ = 0.000, $F_{TSeNS2/control}$ = 1,055.315, $p_{TSeNS2/control}$ = 0.000, $F_{TSeNS4/control}$ = 834.293, $p_{TSeNS4/control}$ = 0.000, $F_{TSeNS6/control}$ = 688.818, $p_{TSeNS6/control}$ = 0.000, $F_{TSeNS10/control}$ = 670.290, $p_{TSeNS10/control}$ = 0.000, $F_{TSeSS2/AA}$ = 2,713.554, $p_{TSeSS2/AA}$ = 0.000, $F_{TSeSS4/AA}$ = 2,399.792, $p_{TSeSS4/AA}$ = 0.000, $F_{TSeSS6/AA}$ = 1,165.621, $p_{TSeSS6/AA}$ = 0.000, $F_{TSeSS10/AA}$ = 795.356, $p_{TSeSS10/AA}$ = 0.000, $F_{TSeNS2/AA}$ = 1,054.379, $p_{TSeNS2/AA}$ = 0.000, $F_{TSeNS4/AA}$ = 834.069, $p_{TSeNS4/AA}$ = 0.000, $F_{TSeNS6/AA}$ = 688.728, $p_{TSeNS6/AA}$ = 0.000, $F_{TSeNS10/AA}$ = 670.247, $p_{TSeNS10/AA}$ = 0.000, $F_{TSeSS2/CTS}$ = 2,712.414, $p_{TSeSS2/CTS}$ = 0.000, $F_{TSeSS4/CTS}$ = 2,398.997, $p_{TSeSS4/CTS}$ = 0.000, $F_{TSeSS6/CTS}$ = 1,165.311, $p_{TSeSS6/CTS}$ = 0.000, $F_{TSeSS10/CTS}$ = 795.227, $p_{TSeSS10/CTS}$ = 0.000, $F_{TSeNS2/CTS}$ = 1,053.559, $p_{TSeNS2/CTS}$ = 0.000, $F_{TSeNS4/CTS}$ = 883.673, $p_{TSeNS4/CTS}$ = 0.000, $F_{TSeNS6/CTS}$ = 688.510, $p_{TSeNS6/CTS}$ = 0.000, $F_{TSeNS10/CTS}$ = 670.120, $p_{TSeNS10/CTS}$ = 0.000, $F_{TSeSS2/CTS+AA}$ = 2,713.269, $p_{TSeSS2/CTS+AA}$ = 0.000, $F_{TSeSS4/CTS+AA}$ = 2,398.719, $p_{TSeSS4/CTS+AA}$ = 0.000, $F_{TSeSS6/CTS+AA}$ = 1,165.106, $p_{TSeSS6/CTS+AA}$ = 0.000, $F_{TSeSS10/CTS+AA}$ = 795.132, $p_{TSeSS10/CTS+AA}$ = 0.000, $F_{TSeNS2/CTS+AA}$ = 1,053.293, $p_{TSeNS2/CTS+AA}$ = 0.000, $F_{TSeNS4/CTS+AA}$ = 883.427, $p_{TSeNS4/CTS+AA}$ = 0.000, $F_{TSeNS6/CTS+AA}$ = 688.357, $p_{TSeNS6/CTS+AA}$ = 0.000, $F_{TSeNS10/CTS+AA}$ = 670.026, $p_{TSeNS10/CTS+AA}$ = 0.000; $F_{ISeSS2/control,AA,CTS,CTS+AA}$ = 1,104.729, $p_{ISeSS2/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeSS4/control,AA,CTS,CTS+AA}$ = 1,296.284, $p_{ISeSS4/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeSS6/control,AA,CTS,CTS+AA}$ = 1,263.680, $p_{ISeSS6/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeSS10/control,AA,CTS,CTS+AA}$ = 1,293.428, $p_{ISeSS10/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeNS2/control,AA,CTS,CTS+AA}$ = 958.820, $p_{ISeNS2/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeNS4/control,AA,CTS,CTS+AA}$ = 2,075.485, $p_{ISeNS4/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeNS6/control,AA,CTS,CTS+AA}$ = 776.227, $p_{ISeNS6/control,AA,CTS,CTS+AA}$ = 0.000, $F_{ISeNS10/control,AA,CTS,CTS+AA}$ = 1,938.764, $p_{ISeNS10/control,AA,CTS,CTS+AA}$ = 0.000; $F_{OSeSS2/control}$ = 1,432.803, $p_{OSeSS2/control}$ = 0.000, $F_{OSeSS4/control}$ = 1,846.244, $p_{OSeSS4/control}$ = 0.000, $F_{OSeSS6/control}$ = 3,444.751, $p_{OSeSS6/control}$ = 0.000, $F_{OSeSS10/control}$ = 1,293.491, $p_{OSeSS10/control}$ = 0.000, $F_{OSeNS2/control}$ = 681.16, $p_{OSeNS2/control}$ = 0.000, $F_{OSeNS4/control}$ = 740.465, $p_{OSeNS4/control}$ = 0.000, $F_{OSeNS6/control}$ = 759.536, $p_{OSeNS6/control}$ = 0.000, $F_{OSeNS10/control}$ = 594.862, $p_{OSeNS10/control}$ = 0.000, $F_{OSeSS2/AA}$ = 1,431.408, $p_{OSeSS2/AA}$ = 0.000, $F_{OSeSS4/AA}$ = 1,845.677, $p_{OSeSS4/AA}$ = 0.000, $F_{OSeSS6/AA}$ = 3,443.892, $p_{OSeSS6/AA}$ = 0.000, $F_{OSeSS10/AA}$ = 1,293.407, $p_{OSeSS10/AA}$ = 0.000, $F_{OSeNS2/AA}$ = 680.714, $p_{OSeNS2/AA}$ = 0.000, $F_{OSeNS4/AA}$ = 740.281, $p_{OSeNS4/AA}$ = 0.000, $F_{OSeNS6/AA}$ = 759.435, $p_{OSeNS6/AA}$ = 0.000, $F_{OSeNS10/AA}$ = 594.825, $p_{OSeNS10/AA}$ = 0.000, $F_{OSeSS2/CTS}$ = 1,430.682, $p_{OSeSS2/CTS}$ = 0.000, $F_{OSeSS4/CTS}$ = 1,844.993, $p_{OSeSS4/CTS}$ = 0.000, $F_{OSeSS6/CTS}$ = 3,443.073, $p_{OSeSS6/CTS}$ = 0.000, $F_{OSeSS10/CTS}$ = 1,293.180, $p_{OSeSS10/CTS}$ = 0.000, $F_{OSeNS2/CTS}$ = 680.103, $p_{OSeNS2/CTS}$ = 0.000, $F_{OSeNS4/CTS}$ = 739.905, $p_{OSeNS4/CTS}$ = 0.000, $F_{OSeNS6/CTS}$ = 759.179, $p_{OSeNS6/CTS}$ = 0.000, $F_{OSeNS10/CTS}$ = 594.701, $p_{OSeNS10/CTS}$ = 0.000, $F_{OSeSS2/CTS+AA}$ = 1,430.501, $p_{OSeSS2/CTS+AA}$ = 0.000, $F_{OSeSS4/CTS+AA}$ = 1,844.688, $p_{OSeSS4/CTS+AA}$ = 0.000, $F_{OSeSS6/CTS+AA}$ = 3,442.806, $p_{OSeSS6/CTS+AA}$ = 0.000, $F_{OSeSS10/CTS+AA}$ = 1,293.023, $p_{OSeSS10/CTS+AA}$ = 0.000, $F_{OSeNS2/CTS+AA}$ = 679.798, $p_{OSeNS2/CTS+AA}$ = 0.000, $F_{OSeNS4/CTS+AA}$ = 739.668, $p_{OSeNS4/CTS+AA}$ = 0.000, $F_{OSeNS6/CTS+AA}$ = 759.003, $p_{OSeNS6/CTS+AA}$ = 0.000, $F_{OSeNS10/CTS+AA}$ = 594.610, $p_{OSeNS10/CTS+AA}$

= 0.000) (Table 1). Meanwhile, the OSe conversion rates of all soy sauce were over 90%. Recently, Gao et al. (1) claimed that the TSe content of soy sauce prepared using selenium-enriched soybean was 79.3 μg/kg, which was far lower than that in the above selenium-enriched soy sauces, suggesting that soy sauces prepared by soybean soaking in nano-selenium solution was a more effective method to prepare selenium-enriched soy sauce. Notably, the contents of TSe, ISe and OSe in NS4, NS6 and NS10 were markedly lower than those in SS4, SS6 and SS10 ($F_{TSeNS4/SS4}$ = 29.278, $p_{TSeNS4/SS4}$ = 0.006, $F_{ISeNS4/SS4}$ = 41.874, $p_{ISeNS4/SS4}$ = 0.003, $F_{OSeNS4/SS4}$ = 24.219, $p_{OSeNS4/SS4}$ = 0.008; $F_{TSeNS6/SS6}$ = 27.386, $p_{TSeNS6/SS6}$ = 0.006, $F_{ISeNS6/SS6}$ = 28.486, $p_{ISeNS6/SS6}$ = 0.007, $F_{OSeNS6/SS6}$ = 14.699, $p_{OSeNS6/SS6}$ = 0.019; $F_{TSeNS10/SS10}$ = 8.348, $p_{TSeNS10/SS10}$ = 0.045, $F_{ISeNS10/SS106}$ = 27.638, $p_{ISeNS10/SS10}$ = 0.006, $F_{OSeNS10/SS10}$ = 9.217, $p_{OSeNS10/SS10}$ = 0.039), respectively, suggesting that sodium selenite was more conducive to being absorbed and transformed into organic selenium by soybean. Because sodium selenite had strong toxicity, selenium-enriched soy sauce prepared by nano-selenium was more acceptable due to its high safety. Furthermore, the contents of TSe, ISe and OSe in the control, AA, CTS and AA+CTS groups were low, which had no significant differences amongst the soy sauces ($F_{TSecontrol/AA}$ = 0.007, $p_{TSecontrol/AA}$ = 0.935, $F_{TSecontrol/CTS}$ = 0.196, $p_{TSecontrol/CTS}$ = 0.681, $F_{TSecontrol/AA+CTS}$ = 0.677, $p_{TSecontrol/AA+CTS}$ = 0.457, $F_{TSeAA/CTS}$ = 0.096, $p_{TSeAA/CTS}$ = 0.772, $F_{TSeAA/AA+CTS}$ = 0.376, $p_{TSeAA/AA+CTS}$ = 0.573, $F_{TSeCTS/AA+CTS}$ = 0.080, $p_{TSeCTS/AA+CTS}$ = 0.791; $F_{OSecontrol/AA}$ = 0.007, $p_{OSecontrol/AA}$ = 0.935, $F_{OSecontrol/CTS}$ = 0.196, $p_{OSecontrol/CTS}$ = 0.681, $F_{OSecontrol/AA+CTS}$ = 0.677, $p_{OSecontrol/AA+CTS}$ = 0.457, $F_{OSeAA/CTS}$ = 0.096, $p_{OSeAA/CTS}$ = 0.772, $F_{OSeAA/AA+CTS}$ = 0.376, $p_{OSeAA/AA+CTS}$ = 0.573, $F_{OSeCTS/AA+CTS}$ = 0.080, $p_{OSeCTS/AA+CTS}$ = 0.791).

The nano-selenium and sodium selenite were firstly adsorbed and were partially transformed into organic selenium by soybean during soaking. Part of the left nano-selenium and sodium selenite were then adsorbed and transformed into organic selenium by *A. oryze* during koji preparation and salt-tolerant bacterial during moromi fermentation. Finally, organic selenium was released into soy sauce by the degraded soybean proteins and autolyzed microorganism (10–12, 28, 29). This might be the possible reason for the high organic selenium conversion rates obtained in both selenium-enriched soy sauces.

Influence of selenium on the antioxidant activities of soy sauces

As shown in Table 1, the DPPH radical scavenging activity (DRSA), ABTS radical scavenging activity (ARSA), reducing

TABLE 1 Selenium contents and antioxidant activities of soy sauces.

	Total Se (μg/kg)	Inorganic Se (μg/kg)	Organic Se (μg/kg)	Organic selenium conversion rate (%)	DPPH radical scavenging activity (μg AAE/mL)	ABTS radical scavenging activity (μmol TE/mL)	Reducing power (μg AAE/mL)	Metal ion chelating activity (μg EE/mL)
Control	5.61 ± 0.29^{h}	0^{h}	5.61 ± 0.29^{h}	100^{a}	769.25 ± 25.05^{c}	56.48 ± 1.96^{d,e}	2,693.72 ± 92.10^{d}	229.24 ± 6.99^{e}
AA	5.63 ± 0.37^{h}	0^{h}	5.63 ± 0.37^{h}	100^{a}	771.03 ± 23.73^{c}	56.99 ± 1.71^{d,e}	2,700.36 ± 83.29^{d}	231.36 ± 6.39^{d,e}
CTS	5.72 ± 0.34^{h}	0^{h}	5.72 ± 0.34^{h}	100^{a}	775.28 ± 24.96^{c}	57.03 ± 1.41^{d,e}	2,721.68 ± 111.18^{d}	235.31 ± 6.27^{d,e}
AA+CTS	5.79 ± 0.26^{h}	0^{h}	5.79 ± 0.26^{h}	100^{a}	779.75 ± 20.56^{b,c}	57.86 ± 1.80^{d,e}	2,732.45 ± 105.37^{d}	237.68 ± 6.84^{d,e}
SS2	216.15 ± 6.99^{g}	11.13 ± 0.58^{g}	205.02 ± 9.12^{g}	94.85 ± 1.15^{b}	784.36 ± 25.78^{b,c}	57.12 ± 1.25^{d,e}	2,713.53 ± 81.01^{d}	240.78 ± 5.28^{d,e}
SS4	427.46 ± 14.91^{e}	25.36 ± 1.22^{e}	402.10 ± 15.98^{e}	94.07 ± 0.46^{b,c}	807.61 ± 16.91^{a,b,c}	59.71 ± 2.21^{c,d}	2,863.17 ± 102.18^{c,d}	265.34 ± 7.05^{c}
SS6	632.89 ± 31.82^{c}	47.41 ± 2.31^{c}	585.48 ± 17.11^{c}	92.51 ± 1.95^{d,e}	829.74 ± 23.53^{a,b}	63.84 ± 2.05^{a,b}	3,049.28 ± 100.29^{a,b}	279.93 ± 7.41^{b}
SS10	1079.15 ± 65.93^{a}	100.29 ± 4.83^{a}	978.86 ± 46.87^{a}	90.71 ± 1.20^{f}	770.84 ± 27.70^{c}	55.13 ± 1.62^{e}	2,712.32 ± 107.53^{d}	229.52 ± 6.44^{e}
NS2	182.74 ± 9.44^{g}	8.76 ± 0.49^{g}	173.98 ± 11.17^{g}	95.21 ± 1.20^{b}	786.95 ± 19.24^{b,c}	58.34 ± 2.13^{c,d,e}	2,789.51 ± 77.69^{c,d}	244.67 ± 6.48^{d}
NS4	348.17 ± 20.54^{f}	19.99 ± 0.76^{f}	328.18 ± 20.53^{f}	94.26 ± 0.34^{b,c}	815.77 ± 31.10^{a,b,c}	61.07 ± 1.54^{b,c}	2,940.63 ± 96.63^{b,c}	274.82 ± 8.26^{b,c}
NS6	543.85 ± 35.52^{d}	37.64 ± 2.34^{d}	506.21 ± 31.46^{d}	93.08 ± 0.30^{c,d}	840.40 ± 27.31^{a}	64.79 ± 1.40^{a}	3,136.47 ± 115.68^{a}	293.48 ± 9.55^{a}
NS10	928.48 ± 61.74^{b}	82.62 ± 3.25^{b}	845.86 ± 59.67^{b}	91.10 ± 0.37^{e,f}	773.03 ± 32.47^{c}	57.37 ± 1.37^{d,e}	2,776.34 ± 84.91^{c,d}	239.04 ± 6.80^{d,e}

$^{a-h}$ Different letters in the same column indicate the significant differences ($p < 0.05$); Organic selenium conversion rate = Organic Se/Total Se × 100%.

power (RP) and metal ion chelating activity (MICA) of soy sauces were 769.25–840.40 μg AAE/mL, 55.13–64.79 μmol TE/mL, 2,693.72–3,136.47 μg AAE/mL and 229.24–293.48 μg EE/mL, respectively, the above four antioxidant activities increased with the enhancement of selenium concentrations from 0 mg/L to 6 mg/L, then decreased with the enhanced selenium concentrations from 6 mg/L to 10 mg/L. The metal ion chelating activity and reducing power of ordinary soy sauce were previously reported as respectively 120.32 μg EE/mL and 2,239.87 μg AAE/mL (7), which were lower than the corresponding values of control determined in the present investigation. Higher ratio (4:1) of saline and koji used in the previous study led to the differences in the antioxidant activities between these two soy sauces. ANOVA indicated that ABTS radical scavenging activity, DPPH radical scavenging activity, metal ion chelating activity and reducing power of SS6 and NS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{ABTSSS6/control} = 20.202$, $p_{ABTSSS6/control} = 0.011$, $F_{ABTSNS6/control} = 35.709$, $p_{ABTSNS6/control} = 0.004$, $F_{ABTSSS6/AA} = 19.752$, $p_{ABTSSS6/AA} = 0.011$, $F_{ABTSNS6/AA} = 37.370$, $p_{ABTSNS6/AA} = 0.004$, $F_{ABTSSS6/CTS} = 22.474$, $p_{ABTSSS6/CTS} = 0.009$, $F_{ABTSNS6/CTS} = 45.757$, $p_{ABTSNS6/CTS} = 0.002$, $F_{ABTSSS6/CTS+AA} = 14.415$, $p_{ABTSSS6/CTS+AA} = 0.019$, $F_{ABTSNS6/CTS+AA} = 27.707$, $p_{ABTSNS6/CTS+AA} = 0.006$; $F_{DPPHSS6/control} = 9.290$, $p_{DPPHSS6/control} = 0.038$, $F_{DPPHNS6/control} = 11.058$, $p_{DPPHNS6/control} = 0.029$, $F_{DPPHSS6/AA} = 9.255$, $p_{DPPHSS6/AA} = 0.038$, $F_{DPPHNS6/AA} = 11.029$, $p_{DPPHNS6/AA} = 0.030$, $F_{DPPHSS6/CTS} = 7.560$, $p_{DPPHSS6/CTS} = 0.049$, $F_{DPPHNS6/CTS} = 9.294$, $p_{DPPHNS6/CTS} = 0.038$, $F_{DPPHSS6/CTS+AA} = 7.675$, $p_{DPPHSS6/CTS+AA} = 0.049$, $F_{DPPHNS6/CTS+AA} = 9.444$, $p_{DPPHNS6/CTS+AA} = 0.037$; $F_{MICSS6/control} = 74.285$, $p_{MICSS6/control} = 0.001$, $F_{MICNS6/control} = 88.391$, $p_{MICNS6/control} = 0.001$, $F_{MICSS6/AA} = 73.920$, $p_{MICSS6/AA} = 0.001$, $F_{MICNS6/AA} = 87.697$, $p_{MICNS6/AA} = 0.001$, $F_{MICSS6/CTS} = 63.392$, $p_{MICSS6/CTS} = 0.001$, $F_{MICNS6/CTS} = 77.778$, $p_{MICNS6/CTS} = 0.001$, $F_{MICSS6/CTS+AA} = 52.660$, $p_{MICSS6/CTS+AA} = 0.002$, $F_{MICNS6/CTS+AA} = 67.694$, $p_{MICNS6/CTS+AA} = 0.001$; $F_{RPSS6/control} = 20.456$, $p_{RPSS6/control} = 0.011$, $F_{RPNS6/control} = 26.897$, $p_{RPNS6/control} = 0.007$, $F_{RPSS6/AA} = 21.490$, $p_{RPSS6/AA} = 0.010$, $F_{RPNS6/AA} = 28.081$, $p_{RPNS6/AA} = 0.006$, $F_{RPSS6/CTS} = 14.361$, $p_{RPSS6/CTS} = 0.019$, $F_{RPNS6/CTS} = 25.050$, $p_{RPNS6/CTS} = 0.011$, $F_{RPSS6/CTS+AA} = 14.231$, $p_{RPSS6/CTS+AA} = 0.020$, $F_{RPNS6/CTS+AA} = 20.000$, $p_{RPNS6/CTS+AA} = 0.011$) and higher than those of SS2, SS4, SS10, NS2, NS4 and NS10 to some extent, but there was no significant differences in the antioxidant activities between SS6 and NS6 ($F_{ABTS} = 0.439$, $p_{ABTS} = 0.544$, $F_{DPPH} = 0.262$, $p_{DPPH} = 0.636$, $F_{MIC} = 3.770$, $p_{MIC} = 0.124$, $F_{RP} = 0.973$, $p_{RP} = 0.380$). The above results demonstrated that sodium selenite and nano-selenium with suitable concentration were capable of enhancing the soy sauce' antioxidant activities.

Wang et al. (4) claimed that the DPPH radical scavenging activity of fermented *Pleurotus eryngii* treated with selenium-enriched *Lactobacillus plantarum* at an initial level of 1×10^9 CFU/mL was significantly increased by 107.33% compared with its control, suggesting that selenium played a positive role during *P. eryngii* fermentation. Ekumah et al. (5) found that the ABTS radical scavenging activity, DPPH radical scavenging activity and reducing power of mulberry wine supplemented with 100 μg selenium-enriched yeast/300 mL mulberry juice was markedly increased by 21.99, 12.06, and 3.20% compared with the control. Selenium had a low pKa (5.2), it could be used as a reducing agent in the experiment of reducing power (30), so that 99% of selenium was deprotonated (31), this further led to stronger nucleophilicity of selenium compounds and enhanced electron donor to reduce Fe^{3+}. Lv et al. (32) proved that enzymatic hydrolysate of soybean had an iron chelating activity and speculated that the binding site between soybean protein hydrolysate and iron was the carboxyl group of Glu and Asp residues. Soy sauce contained abundant soybean peptides, which might contribute to the strong iron chelating activity. But the detailed reasons caused the significant differences in the antioxidant activities amongst the soy sauces needed further analyses.

Analyses of proximate indices

As demonstrated in Table 2, the formaldehyde nitrogen (FN), reducing sugar (RS), total sugar (TS) and non-salt soluble solids (NSS) contents of soy sauces increased with the enhancement of selenium concentrations from 0 to 6 mg/L, then decreased with the enhanced selenium concentrations from 6 to 10 mg/L, which were 0.85–0.94 g/100 mL, 2.33–2.85 g/100 mL, 3.05–3.71 g/100 mL, and 156.45–181.45 g/L. The FN, RS, TS and NSS contents of ordinary soy sauce were previously reported to be 0.54 g/100 mL, 2.69 g/100 mL, 2.97 g/100 mL and 93.00 g/L, respectively (8), which were lower than the corresponding values except RS content of the control determined in the present investigation. Higher ratio (4:1) of saline and koji used in the previous study led to the differences in the above indices between these two soy sauces.

ANOVA indicated that FN, RS, TS and NSS contents of SS6 and NS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{FNSS6/control} = 16.333$, $p_{FNSS6/control} = 0.016$, $F_{FNNS6/control} = 60.750$, $p_{FNNS6/control} = 0.001$, $F_{FNSS6/AA} = 16.333$, $p_{FNSS6/AA} = 0.016$, $F_{FNNS6/AA} = 60.750$, $p_{FNNS6/AA} = 0.001$, $F_{FNSS6/CTS} = 12.000$, $p_{FNSS6/CTS} = 0.026$, $F_{FNNS6/CTS} = 48.000$, $p_{FNNS6/CTS} = 0.002$, $F_{FNSS6/CTS+AA} = 8.308$, $p_{FNSS6/CTS+AA} = 0.045$, $F_{FNNS6/CTS+AA} = 24.000$, $p_{FNNS6/CTS+AA} = 0.008$; $F_{RSSS6/control} = 74.682$, $p_{RSSS6/control} = 0.001$, $F_{RSNS6/control} = 44.025$, $p_{RSNS6/control} = 0.003$, $F_{RSSS6/AA} = 58.541$,

TABLE 2 Proximate indices of soy sauces.

	Formaldehyde nitrogen (g/100 mL)	Total titratable acid (g/100 mL)	Reducing sugar (g/100 mL)	Total sugar (g/100 mL)	Maillard reaction products	Non-salt soluble solids (g/L)	Total phenolics (mg GAE/mL)	Total flavonoids (mg RE/mL)
Control	0.85 ± 0.02^{c}	$1.09 \pm 0.02^{d,e}$	2.37 ± 0.06^{e}	3.07 ± 0.10^{f}	7.55 ± 0.32^{d}	158.48 ± 5.14^{c}	2.07 ± 0.12^{d}	0.22 ± 0.01^{c}
AA	0.85 ± 0.03^{c}	$1.08 \pm 0.03^{d,e}$	2.40 ± 0.05^{e}	3.05 ± 0.08^{f}	7.57 ± 0.24^{d}	157.86 ± 4.89^{c}	2.08 ± 0.15^{d}	0.22 ± 0.01^{c}
CTS	0.86 ± 0.02^{c}	$1.09 \pm 0.03^{d,e}$	$2.45 \pm 0.06^{d,e}$	$3.15 \pm 0.14^{e,f}$	7.61 ± 0.37^{d}	159.11 ± 5.43^{c}	$2.12 \pm 0.13^{c,d}$	0.22 ± 0.01^{c}
AA+CTS	0.86 ± 0.02^{c}	$1.11 \pm 0.05^{c,d,e}$	$2.45 \pm 0.10^{d,e}$	$3.16 \pm 0.14^{d,e,f}$	7.66 ± 0.35^{d}	159.37 ± 6.26^{c}	$2.12 \pm 0.09^{c,d}$	0.22 ± 0.01^{c}
SS2	$0.87 \pm 0.04^{b,c}$	$1.15 \pm 0.04^{c,d}$	$2.59 \pm 0.09^{c,d}$	$3.30 \pm 0.11^{c,d,e}$	7.83 ± 0.20^{d}	$164.23 \pm 5.75^{b,c}$	$2.19 \pm 0.12^{c,d}$	$0.23 \pm 0.01^{b,c}$
SS4	$0.89 \pm 0.03^{a,b,c}$	1.29 ± 0.05^{b}	$2.70 \pm 0.10^{b,c}$	$3.48 \pm 0.13^{b,c}$	$8.17 \pm 0.40^{c,d}$	$171.79 \pm 6.55^{a,b}$	$2.35 \pm 0.15^{b,c}$	0.24 ± 0.01^{b}
SS6	$0.92 \pm 0.03^{a,b}$	1.40 ± 0.04^{a}	$2.83 \pm 0.07^{a,b}$	$3.64 \pm 0.10^{a,b}$	$8.63 \pm 0.34^{b,c}$	179.72 ± 5.69^{a}	2.61 ± 0.07^{a}	0.27 ± 0.01^{a}
SS10	0.85 ± 0.04^{c}	1.05 ± 0.04^{e}	2.33 ± 0.05^{e}	$3.08 \pm 0.09^{e,f}$	7.51 ± 0.38^{d}	156.45 ± 5.31^{c}	2.09 ± 0.11^{d}	0.22 ± 0.01^{c}
NS2	$0.88 \pm 0.02^{b,c}$	1.18 ± 0.03^{c}	2.60 ± 0.07^{c}	$3.37 \pm 0.13^{c,d}$	7.98 ± 0.36^{d}	$163.96 \pm 5.42^{b,c}$	$2.28 \pm 0.11^{b,c,d}$	$0.23 \pm 0.01^{b,c}$
NS4	$0.90 \pm 0.03^{a,b,c}$	1.32 ± 0.06^{b}	$2.75 \pm 0.09^{a,b}$	$3.50 \pm 0.13^{a,b,c}$	$8.84 \pm 0.43^{a,b}$	$171.37 \pm 6.51^{a,b}$	$2.44 \pm 0.15^{a,b}$	0.24 ± 0.01^{b}
NS6	0.94 ± 0.02^{a}	1.44 ± 0.05^{a}	2.85 ± 0.11^{a}	3.71 ± 0.15^{a}	9.40 ± 0.47^{a}	181.45 ± 6.23^{a}	2.64 ± 0.10^{a}	0.28 ± 0.01^{a}
NS10	$0.87 \pm 0.03^{b,c}$	$1.08 \pm 0.04^{d,e}$	2.37 ± 0.08^{e}	$3.13 \pm 0.11^{e,f}$	7.59 ± 0.24^{d}	160.55 ± 5.87^{c}	$2.13 \pm 0.15^{c,d}$	0.22 ± 0.01^{c}

$^{a-f}$Different letters in the same column indicate the significant differences ($p < 0.05$); the contents of ascorbic acid (40 μg/mL) and chitosan (9 μg/mL) in AA, CTS and AA+CTS are the same as those in NS6.

$p_{RSSS6/AA} = 0.002$, $F_{RSNS6/AA} = 32.877$, $p_{RSNS6/AA} = 0.005$, $F_{RSSS6/CTS} = 50.965$, $p_{RSSS6/CTS} = 0.002$, $F_{RSNS6/CTS} = 30.573$, $p_{RSNS6/CTS} = 0.005$, $F_{RSSS6/CTS+AA} = 15.785$, $p_{RSSS6/CTS+AA} = 0.016$, $F_{RSNS6/CTS+AA} = 12.217$, $p_{RSNS6/CTS+AA} = 0.025$; $F_{TSSS6/control} = 48.735$, $p_{TSSS6/control} = 0.002$, $F_{TSNS6/control} = 37.809$, $p_{TSNS6/control} = 0.004$, $F_{TSSS6/AA} = 63.677$, $p_{TSSS6/AA} = 0.001$, $F_{TSNS6/AA} = 45.218$, $p_{TSNS6/AA} = 0.003$, $F_{TSSS6/CTS} = 24.334$, $p_{TSSS6/CTS} = 0.008$, $F_{TSNS6/CTS} = 22.347$, $p_{TSNS6/CTS} = 0.009$, $F_{TSSS6/CTS+AA} = 23.351$, $p_{TSSS6/CTS+AA} = 0.008$, $F_{TSNS6/CTS+AA} = 21.556$, $p_{TSNS6/CTS+AA} = 0.010$; $F_{NSSSS6/control} = 23.019$, $p_{NSSSS6/control} = 0.009$, $F_{NSSNS6/control} = 24.265$, $p_{NSSNS6/control} = 0.008$, $F_{NSSSS6/AA} = 25.469$, $p_{NSSSS6/AA} = 0.007$, $F_{NSSNS6/AA} = 26.616$, $p_{NSSNS6/AA} = 0.007$, $F_{NSSSS6/CTS} = 20.600$, $p_{NSSSS6/CTS} = 0.011$, $F_{NSSNS6/CTS} = 21.992$, $p_{NSSNS6/CTS} = 0.009$, $F_{NSSSS6/CTS+AA} = 17.360$, $p_{NSSSS6/CTS+AA} = 0.014$, $F_{NSSNS6/CTS+AA} = 18.751$, $p_{NSSNS6/CTS+AA} = 0.012$) and higher than those of SS2, SS4, SS10, NS2, NS4 and NS10 to some extent, but there was no significant differences in the FN, RS, TS and NSS contents between SS6 and NS6 ($F_{FN} = 0.923$, $p_{FN} = 0.391$, $F_{RS} = 0.071$, $p_{RS} = 0.804$, $F_{TS} = 0.452$, $p_{TS} = 0.538$, $F_{NSS} = 0.126$, $p_{NSS} = 0.740$). The above results demonstrated that sodium selenite and nano-selenium with suitable concentration were capable of enhancing the FN, RS, TS and NSS contents of soy sauce. FN, RS, TS were the enzymatically hydrolyzed products of proteins and starch catalyzed by proteases, amylase and glucamylase during koji fermentation and moromi fermentation, and peptides, free amino acids, polysaccharides, monosaccharides, fats, etc. constitute NSS of soy sauce (7, 8). The materials, ratios of materials and manufacturing process were the same, thus the differences in FN, RS, TS and NSS contents between these two soy sauces might be caused by the differences in enzyme activities between these two soy sauces.

The change in total titratable acidity (TTA) content of soy sauces along with the increase of selenium content was similar to those of FN, RS, TS and NSS contents. The TTA contents of NS6 and SS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{SS6/control} = 144.150$, $p_{SS6/control} = 0.000$, $F_{NS6/control} = 126.724$, $p_{NS6/control} = 0.000$, $F_{SS6/AA} = 122.880$, $p_{SS6/AA} = 0.000$, $F_{NS6/AA} = 114.353$, $p_{NS6/AA} = 0.000$, $F_{SS6/CTS} = 115.320$, $p_{SS6/CTS} = 0.000$, $F_{NS6/CTS} = 108.088$, $p_{NS6/CTS} = 0.000$, $F_{SS6/CTS+AA} = 61.537$, $p_{SS6/CTS+AA} = 0.001$, $F_{NS6/CTS+AA} = 65.350$, $p_{NS6/CTS+AA} = 0.001$) and higher than those of SS2, SS4, SS10, NS2, NS4 and NS10 to some extent, but there was no marked difference in the TTA contents between SS6 and NS6 ($F = 1.171$, $p = 0.340$). Organic acids in soy sauce were the products of microbial metabolism (mainly lactic acid bacteria). An appropriate amount of selenium could promote the growth of microorganisms, while high concentration of selenium might inhibit their growth (1, 33). The change trend of microorganism number along with selenium content was consistent with the

change trend of total titratable acidity content observed in Table 2.

The change in Maillard reaction products content of soy sauces along with the increased selenium content was similar to those of FN, RS, TS, NSS and TTA contents. The Maillard reaction products contents of NS6 and SS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{SS6/control} = 16.051$, $p_{SS6/control} = 0.016$, $F_{NS6/control} = 31.758$, $p_{NS6/control} = 0.005$, $F_{SS6/AA} = 19.462$, $p_{SS6/AA} = 0.012$, $F_{NS6/AA} = 36.074$, $p_{NS6/AA} = 0.004$, $F_{SS6/CTS} = 12.361$, $p_{SS6/CTS} = 0.025$, $F_{NS6/CTS} = 26.865$, $p_{NS6/CTS} = 0.007$, $F_{SS6/CTS+AA} = 11.855$, $p_{SS6/CTS+AA} = 0.026$, $F_{NS6/CTS+AA} = 26.450$, $p_{NS6/CTS+AA} = 0.007$). The Maillard reaction products were a kind of complex compounds formed *via* carbonyl-amine condensation between reducing sugars and carbonyl compounds and additional reactions, which were important components of soy sauce pigment, flavor and antioxidant compounds (7, 34). As mentioned above, selenium-enriched soy sauces contained more abundant free amino acids and reducing sugars, which would inevitably lead to form more Maillard reaction products and stronger antioxidant activities of selenium-enriched soy sauces.

Total flavonoids and total phenolics contents

As shown in Table 2, the total flavonoids (TF) and total phenolics (TP) contents of soy sauces increased with the enhancement of selenium concentrations from 0 to 6 mg/L, then decreased with the enhanced selenium concentrations from 6 to 10 mg/L, which were 0.22–0.28 mg RE/mL and 2.07–2.64 mg GAE/mL. ANOVA indicated that TF and TP contents of SS6 and NS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{TFSS6/control} = 45.326$, $p_{TFSS6/control} = 0.003$, $F_{TFNS6/control} = 39.947$, $p_{TFNS6/control} = 0.003$, $F_{TFSS6/AA} = 30.755$, $p_{TFSS6/AA} = 0.005$, $F_{TFNS6/AA} = 28.948$, $p_{TFNS6/AA} = 0.006$, $F_{TFSS6/CTS} = 33.041$, $p_{TFSS6/CTS} = 0.005$, $F_{TFNS6/CTS} = 30.156$, $p_{TFNS6/CTS} = 0.005$, $F_{TFSS6/CTS+AA} = 55.408$, $p_{TFSS6/CTS+AA} = 0.002$, $F_{TFNS6/CTS+AA} = 44.818$, $p_{TFNS6/CTS+AA} = 0.003$; $F_{TPSS6/control} = 37.500$, $p_{TPSS6/control} = 0.004$, $F_{TPNS6/control} = 54.000$, $p_{TPNS6/control} = 0.002$, $F_{TPSS6/AA} = 37.500$, $p_{TPSS6/AA} = 0.004$, $F_{TPNS6/AA} = 54.000$, $p_{TPNS6/AA} = 0.002$, $F_{TPSS6/CTS} = 37.500$, $p_{TPSS6/CTS} = 0.004$, $F_{TPNS6/CTS} = 54.000$, $p_{TPNS6/CTS} = 0.002$, $F_{TPSS6/CTS+AA} = 37.500$, $p_{TPSS6/CTS+AA} = 0.004$, $F_{TPNS6/CTS+AA} = 54.000$, $p_{TPNS6/CTS+AA} = 0.002$) and higher than those of SS2, SS4, SS10, NS2, NS4 and NS10 to some extent, but there was no notable differences found in the TF and TP contents between SS6 and NS6 ($F_{TFSS6/NS6} = 0.181$, $p_{TFSS6/NS6} = 0.692$; $F_{TPSS6/NS6} = 1.500$, $p_{TPSS6/NS6} = 0.288$).

TP in soy sauces prepared in this work was greater than that in the citrus peel koji soy sauce (35) and the soy sauce treated with ultrasound (7), and TF in soy sauces prepared in this work was higher than that of soy sauce reported by Gao et al. (7). Different raw materials, manufacturing process and NaCl concentration might cause the above marked differences in TP and TF contents. However, the content differences in TP and TF amongst soy sauces prepared in this work needed to be explored further.

Free amino acids

As shown in Table 3, whether sodium selenite or nano-selenium treated soy sauces, the free amino acids (FAAs) contents of soy sauces increased with the enhancement of selenium concentrations from 0 to 6 mg/L, then decreased with the enhanced selenium concentrations from 6 to 10 mg/L, which ranged from 40.09 to 47.59 mg/mL. ANOVA indicated that FAAs contents of SS6 and NS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{SS6/control} = 20.683$, $p_{SS6/control} = 0.010$, $F_{NS6/control} = 25.917$, $p_{NS6/control} = 0.007$, $F_{SS6/AA} = 20.042$, $p_{SS6/AA} = 0.011$, $F_{NS6/AA} = 25.141$, $p_{NS6/AA} = 0.007$, $F_{SS6/CTS} = 16.563$, $p_{SS6/CTS} = 0.015$, $F_{NS6/CTS} = 21.255$, $p_{NS6/CTS} = 0.010$, $F_{SS6/CTS+AA} = 15.503$, $p_{SS6/CTS+AA} = 0.017$, $F_{NS6/CTS+AA} = 20.097$, $p_{NS6/CTS+AA} = 0.011$) and higher than those of SS2, SS4, SS10, NS2, NS4 and NS10 to some extent, but there was no significant difference in the FAAs contents between SS6 and NS6 ($F = 0.299$, $p = 0.613$). Specifically, the most plentiful amino acid was glutamic acid, followed by aspartic acid in all soy sauces, which were consistent with previous investigations (7, 8).

It was worth mentioning that the contents of cysteine and methionine in soy sauces treated by Na_2SeO_3 and nano-selenium were lower than those in the control, AA, CTS and AA+CTS. In microbial metabolism, the adsorbed selenium is usually converted into selenocysteine, Se-methyselenocysteine, selenomethionine, etc., and the synthesis of organic selenium usually requires the consumption of cysteine and methionine (10–12). By the same token, the contents of cysteine and methionine in the selenium treated soy sauces decreased.

Antioxidant activities of ingredients in NS6

Because NS6 had the strongest antioxidant activities, antioxidant activities of the corresponding soluble soybean polysaccharide, chitosan, ascorbic acid, sodium selenite, nano-selenium and free amino acids were measured to calculate their contributions to the total antioxidant activities of NS6.

TABLE 3 Free amino acid compositions of soy sauces.

FAAs (mg/mL)	Control	AA	CTS	AA+CTS	SS2	SS4	SS6	SS10	NS2	NS4	NS6	NS10
Aspartic acid	$4.08 \pm 0.10^{c,d}$	4.04 ± 0.09^{d}	$4.09 \pm 0.10^{c,d}$	$4.09 \pm 0.10^{c,d}$	$4.17 \pm 0.12^{b,c,d}$	$4.32 \pm 0.13^{a,b,c}$	4.44 ± 0.15^{a}	4.06 ± 0.11^{d}	$4.18 \pm 0.10^{b,c,d}$	$4.37 \pm 0.18^{a,b}$	4.52 ± 0.19^{a}	$4.11 \pm 0.16^{c,d}$
Glutamic acid	6.96 ± 0.20^{a}	6.95 ± 0.23^{a}	6.96 ± 0.22^{a}	6.96 ± 0.22^{a}	7.10 ± 0.28^{a}	7.26 ± 0.26^{a}	7.38 ± 0.29^{a}	6.96 ± 0.18^{a}	7.15 ± 0.20^{a}	7.28 ± 0.30^{a}	7.40 ± 0.29^{a}	6.99 ± 0.26^{a}
Serine	2.48 ± 0.08^{f}	2.49 ± 0.06^{f}	2.48 ± 0.11^{f}	2.51 ± 0.11^{f}	$2.64 \pm 0.08^{e,f}$	$2.88 \pm 0.12^{c,d}$	$3.13 \pm 0.14^{a,b}$	2.43 ± 0.12^{f}	$2.75 \pm 0.11^{d,e}$	$2.98 \pm 0.15^{b,c}$	3.22 ± 0.15^{a}	2.52 ± 0.13^{f}
Histidine	1.56 ± 0.05^{c}	1.54 ± 0.04^{c}	1.55 ± 0.04^{c}	1.55 ± 0.04^{c}	$1.64 \pm 0.04^{b,c}$	1.78 ± 0.07^{a}	1.84 ± 0.05^{a}	1.55 ± 0.03^{c}	1.68 ± 0.07^{b}	1.80 ± 0.08^{a}	1.86 ± 0.06^{a}	1.56 ± 0.04^{c}
Glycine	$1.04 \pm 0.03^{d,e}$	1.03 ± 0.03^{e}	$1.05 \pm 0.04^{d,e}$	$1.05 \pm 0.04^{d,e}$	$1.09 \pm 0.03^{d,e}$	1.20 ± 0.04^{c}	$1.33 \pm 0.05^{a,b}$	1.02 ± 0.04^{e}	1.11 ± 0.03^{d}	1.27 ± 0.04^{b}	1.36 ± 0.05^{a}	$1.08 \pm 0.03^{d,e}$
Threonine	$1.64 \pm 0.06^{e,f}$	1.60 ± 0.04^{f}	$1.69 \pm 0.05^{d,e,f}$	$1.69 \pm 0.05^{d,e,f}$	$1.69 \pm 0.05^{d,e,f}$	$1.78 \pm 0.06^{b,c,d}$	$1.88 \pm 0.07^{a,b}$	1.60 ± 0.06^{f}	$1.74 \pm 0.05^{c,d,e}$	$1.81 \pm 0.07^{b,c}$	1.96 ± 0.09^{a}	$1.64 \pm 0.06^{e,f}$
Arginine	$3.94 \pm 0.13^{d,e}$	$3.94 \pm 0.13^{d,e}$	$4.03 \pm 0.17^{c,d,e}$	$4.08 \pm 0.17^{b,c,d,e}$	$4.14 \pm 0.15^{b,c,d}$	$4.28 \pm 0.13^{a,b,c}$	4.48 ± 0.12^{a}	3.83 ± 0.14^{e}	$4.17 \pm 0.14^{b,c,d}$	$4.34 \pm 0.20^{a,b}$	4.53 ± 0.19^{a}	$3.98 \pm 0.15^{d,e}$
Alanine	$1.88 \pm 0.07^{f,g}$	$1.89 \pm 0.05^{f,g}$	$1.96 \pm 0.08^{e,f,g}$	$1.99 \pm 0.08^{d,e,f}$	$1.98 \pm 0.08^{d,e,f}$	$2.12 \pm 0.07^{c,d}$	$2.30 \pm 0.10^{a,b}$	1.81 ± 0.09^{g}	$2.05 \pm 0.08^{c,d,e}$	$2.18 \pm 0.06^{b,c}$	2.39 ± 0.10^{a}	$1.87 \pm 0.11^{f,g}$
Tyrosine	1.16 ± 0.03^{e}	1.17 ± 0.03^{e}	1.18 ± 0.05^{e}	1.18 ± 0.05^{e}	$1.23 \pm 0.03^{d,e}$	$1.30 \pm 0.04^{c,d}$	$1.40 \pm 0.04^{a,b}$	1.17 ± 0.05^{e}	1.26 ± 0.03^{d}	$1.36 \pm 0.04^{b,c}$	1.44 ± 0.05^{a}	1.17 ± 0.03^{e}
Cysteine	0.08 ± 0.01^{b}	$0.07 \pm 0.01^{b,c}$	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	$0.07 \pm 0.01^{b,c}$	$0.06 \pm 0.01^{c,d}$	$0.04 \pm 0.01^{e,f}$	$0.03 \pm 0.01^{f,g}$	$0.06 \pm 0.01^{c,d}$	$0.05 \pm 0.01^{d,e}$	$0.03 \pm 0.01^{f,g}$	0.02 ± 0.01^{g}
Valine	2.54 ± 0.08^{d}	2.53 ± 0.11^{d}	2.55 ± 0.09^{d}	2.55 ± 0.09^{d}	$2.64 \pm 0.09^{c,d}$	$2.70 \pm 0.10^{b,c,d}$	$2.84 \pm 0.10^{a,b}$	2.53 ± 0.07^{d}	$2.68 \pm 0.11^{b,c,d}$	$2.79 \pm 0.07^{a,b,c}$	2.90 ± 0.13^{a}	2.58 ± 0.06^{d}
Methionine	$0.72 \pm 0.02^{b,c}$	0.74 ± 0.03^{b}	0.79 ± 0.02^{a}	0.79 ± 0.02^{a}	0.70 ± 0.01^{c}	0.65 ± 0.02^{d}	0.58 ± 0.01^{e}	0.46 ± 0.01^{f}	0.62 ± 0.03^{d}	0.56 ± 0.02^{e}	0.48 ± 0.01^{f}	0.40 ± 0.01^{g}
Tryptophan	0.46 ± 0.01^{e}	0.45 ± 0.01^{e}	0.48 ± 0.02^{e}	0.48 ± 0.02^{e}	0.54 ± 0.01^{d}	0.60 ± 0.01^{c}	0.68 ± 0.02^{a}	0.45 ± 0.02^{e}	0.59 ± 0.01^{c}	0.65 ± 0.02^{b}	0.70 ± 0.03^{a}	0.46 ± 0.02^{e}
Phenylalanine	$2.68 \pm 0.08^{d,e}$	$2.70 \pm 0.06^{d,e}$	$2.65 \pm 0.06^{d,e}$	$2.65 \pm 0.06^{d,e}$	$2.79 \pm 0.08^{c,d,e}$	$2.97 \pm 0.07^{b,c}$	$3.14 \pm 0.13^{a,b}$	2.61 ± 0.15^{e}	$2.83 \pm 0.09^{c,d}$	$3.09 \pm 0.10^{a,b}$	3.24 ± 0.15^{a}	$2.65 \pm 0.13^{d,e}$
Isoleucine	1.92 ± 0.07^{d}	$1.93 \pm 0.09^{c,d}$	$1.93 \pm 0.07^{c,d}$	$1.93 \pm 0.07^{c,d}$	$1.98 \pm 0.04^{c,d}$	$2.06 \pm 0.09^{b,c}$	$2.18 \pm 0.08^{a,b}$	1.90 ± 0.05^{d}	$2.03 \pm 0.06^{c,d}$	$2.17 \pm 0.08^{a,b}$	2.24 ± 0.09^{a}	$1.95 \pm 0.06^{c,d}$
Leucine	$3.18 \pm 0.12^{e,f}$	$3.21 \pm 0.09^{d,e,f}$	$3.23 \pm 0.14^{d,e,f}$	$3.23 \pm 0.14^{d,e,f}$	$3.38 \pm 0.12^{c,d,e}$	$3.52 \pm 0.13^{b,c}$	3.81 ± 0.15^{a}	3.13 ± 0.10^{f}	$3.43 \pm 0.11^{c,d}$	$3.66 \pm 0.15^{a,b}$	3.85 ± 0.13^{a}	$3.28 \pm 0.14^{d,e,f}$
Lysine	$3.08 \pm 0.09^{c,d}$	$3.09 \pm 0.09^{c,d}$	$3.11 \pm 0.08^{c,d}$	$3.12 \pm 0.08^{c,d}$	$3.19 \pm 0.10^{b,c,d}$	$3.26 \pm 0.12^{b,c}$	3.36 ± 0.08^{a}	3.05 ± 0.09^{d}	$3.21 \pm 0.07^{b,c,d}$	$3.33 \pm 0.16^{a,b}$	3.46 ± 0.11^{a}	$3.09 \pm 0.10^{c,d}$
Proline	$1.56 \pm 0.06^{f,g,h}$	$1.58 \pm 0.04^{f,g,h}$	$1.58 \pm 0.08^{f,g,h}$	$1.66 \pm 0.08^{e,f,g}$	$1.68 \pm 0.05^{d,e,f}$	$1.79 \pm 0.05^{c,d}$	$1.94 \pm 0.06^{a,b}$	1.50 ± 0.06^{h}	$1.74 \pm 0.05^{d,e}$	$1.87 \pm 0.09^{b,c}$	2.01 ± 0.09^{a}	$1.55 \pm 0.07^{g,h}$
Total	$40.96 \pm 1.20^{d,f}$	$40.95 \pm 1.27^{d,f}$	$41.41 \pm 1.32^{d,f}$	$41.61 \pm 1.30^{d,f}$	$42.65 \pm 1.49^{c,d,f}$	$44.53 \pm 1.52^{a,b,c}$	46.75 ± 1.85^{a}	40.09 ± 1.56^{f}	$43.28 \pm 1.40^{b,c,d}$	$45.56 \pm 2.01^{a,b,c}$	47.59 ± 1.91^{a}	$40.90 \pm 1.56^{d,f}$

[a−h] Different letters in the same row indicate the significant differences ($p < 0.05$).

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TABLE 4 Antioxidant activities of ingredients in NS6.

	Content (μg/mL)	DPPH radical scavenging activity (μg AAE/mL)	ABTS radical scavenging activity (μmol TE/mL)	Reducing power (μg AAE/mL)	Metal ion chelating activity (μg EE/mL)
NS6	-	840.40 ± 27.31^{a}	64.79 ± 1.40^{a}	$3{,}136.47 \pm 115.68^{a}$	293.48 ± 9.55^{a}
Soybean polysaccharide	33,500	4.81 ± 0.18^{d}	0.02 ± 0.01^{d}	10.58 ± 0.36^{d}	nd
Chitosan	9	nd	nd	0.26 ± 0.01^{f}	nd
Ascorbic acid	40	41.42 ± 1.41^{c}	0.19 ± 0.01^{c}	41.61 ± 1.57^{c}	nd
Sodium selenite	13.14	nd	nd	0.89 ± 0.03^{e}	nd
Nano-selenium	6	42.64 ± 2.15^{c}	0.20 ± 0.01^{c}	49.77 ± 2.34^{b}	nd
FAAs mixture of NS6	47,590	67.14 ± 2.88^{b}	23.55 ± 0.84^{b}	54.52 ± 2.50^{b}	10.26 ± 0.39^{b}

$^{a-d}$ Different letters in the same column indicate the significant differences ($p < 0.05$), nd: not detected.

TABLE 5 Effects of selenium on enzyme activities in kojis.

	Acid protease activities (U/g)	Neutral protease activities (U/g)	α-Amylase activities (U/g)	Glucamylase activities (U/g)	Cellulase activities (U/g)	Pectinase activities (U/g)
Control	185.34 ± 5.45^{e}	$1{,}156.52 \pm 33.07^{b}$	165.14 ± 4.24^{d}	$1{,}812.57 \pm 59.25^{e}$	240.17 ± 8.16^{d}	204.43 ± 6.70^{f}
AA	188.08 ± 6.22^{e}	$1{,}161.21 \pm 33.16^{b}$	$167.42 \pm 4.86^{c,d}$	$1{,}819.23 \pm 55.84^{d,e}$	243.56 ± 9.47^{d}	205.27 ± 5.87^{f}
CTS	$190.42 \pm 5.83^{d,e}$	$1{,}163.68 \pm 31.28^{b}$	$168.54 \pm 5.06^{c,d}$	$1{,}836.93 \pm 61.68^{d,e}$	244.40 ± 7.08^{d}	$209.66 \pm 8.14^{e,f}$
AA+CTS	$191.99 \pm 5.16^{d,e}$	$1{,}168.53 \pm 26.73^{b}$	$168.83 \pm 5.39^{c,d}$	$1{,}842.16 \pm 75.31^{c,d,e}$	248.58 ± 7.66^{d}	$210.39 \pm 8.32^{e,f}$
SS2	$196.76 \pm 4.52^{d,e}$	$1{,}175.78 \pm 35.77^{a,b}$	$174.90 \pm 4.34^{c,d}$	$1{,}896.34 \pm 67.99^{c,d,e}$	267.29 ± 10.58^{c}	$217.62 \pm 7.78^{e,f}$
SS4	$209.14 \pm 8.63^{b,c}$	$1{,}202.62 \pm 36.99^{a,b}$	189.28 ± 6.20^{b}	$1{,}948.40 \pm 88.15^{a,b,c,d}$	$282.59 \pm 9.74^{b,c}$	$231.81 \pm 8.19^{c,d}$
SS6	235.06 ± 9.75^{a}	$1{,}240.26 \pm 36.01^{a}$	216.46 ± 8.41^{a}	$2{,}028.69 \pm 70.75^{a,b}$	309.84 ± 10.02^{a}	$252.52 \pm 7.81^{a,b}$
SS10	186.84 ± 4.58^{e}	$1{,}159.96 \pm 37.91^{b}$	166.07 ± 5.31^{d}	$1{,}813.75 \pm 70.20^{e}$	242.27 ± 8.61^{d}	205.80 ± 8.72^{f}
NS2	$200.37 \pm 6.02^{c,d}$	$1{,}181.35 \pm 45.50^{a,b}$	177.58 ± 4.37^{c}	$1{,}914.62 \pm 56.71^{b,c,d,e}$	273.73 ± 7.82^{c}	$223.64 \pm 6.96^{d,e}$
NS4	218.22 ± 5.71^{b}	$1{,}215.67 \pm 48.58^{a,b}$	195.00 ± 5.71^{b}	$1{,}968.94 \pm 65.65^{a,b,c}$	290.90 ± 9.06^{b}	$240.93 \pm 7.38^{b,c}$
NS6	244.60 ± 7.53^{a}	$1{,}269.38 \pm 43.25^{a}$	220.61 ± 8.59^{a}	$2{,}043.15 \pm 77.42^{a}$	314.57 ± 11.89^{a}	256.36 ± 7.32^{a}
NS10	$190.98 \pm 4.87^{d,e}$	$1{,}163.39 \pm 38.75^{a,b}$	$167.34 \pm 4.29^{c,d}$	$1{,}823.47 \pm 63.01^{d,e}$	248.72 ± 8.08^{d}	$211.70 \pm 6.72^{e,f}$

$^{a-f}$ Different letters in the same column indicate the significant differences ($p < 0.05$).

As shown in Table 4, free amino acids had strong ABTS radical scavenging activity, DPPH radical scavenging activity, reducing power and metal ion chelating activity. Secondly, ascorbic acid and nano-selenium had strong reducing power and DPPH radical scavenging activity, and most reducing power and DPPH radical scavenging activity of nano-selenium should attributed to ascorbic acid in nano-selenium. However, due to the long fermentation time of soy sauce, ascorbic acid was easy to be oxidized and inactivated, losing the corresponding antioxidant capacity. Therefore, ascorbic acid and nano-selenium were not the main contributors for the antioxidant activities enhancement of NS6. From the four antioxidant activities of soybean polysaccharide, chitosan, sodium selenite, it could be seen that their contribution to the total antioxidant activities of NS6 were negligible. Therefore, FAAs might play an important role in enhancing the antioxidant activities of NS6.

Effects of selenium on enzyme activities of kojis

As shown in Table 5, whether sodium selenite or nano-selenium treated soy sauces, the activities of neutral protease, acid protease, α-amylase, cellulase, glucoamylase and pectinase in kojis increased with the enhancement of selenium concentrations from 0 to 6 mg/L, then decreased with the enhancement of selenium concentrations from 6 to 10 mg/L, which were 1,156.52–1,269.38 U/g, 185.34–244.60 U/g, 165.14–220.61 U/g, 240.17–314.57 U/g, 1,812.57–2,043.15 U/g, 204.43–256.36 U/g. ANOVA indicated that the neutral protease, acid protease, α-amylase, cellulase, glucoamylase and pectinase activities of SS6 and NS6 were significantly higher than those of the control, AA, CTS and AA+CTS ($F_{neutralproteaseSS6/control} = 8.801$ $p_{neutralproteaseSS6/control} = 0.041$, $F_{neutralproteaseNS6/control}$

$= 12.891$, $p_{neutralproteaseNS6/control} = 0.023$, $F_{neutralproteaseSS6/AA} = 7.823$, $p_{neutralproteaseSS6/AA} = 0.049$, $F_{neutralproteaseNS6/AA} = 11.818$, $p_{neutralproteaseNS6/AA} = 0.026$, $F_{neutralproteaseSS6/CTS} = 7.735$, $p_{neutralproteaseSS6/CTS} = 0.049$, $F_{neutralproteaseNS6/CTS} = 11.765$, $p_{neutralproteaseNS6/CTS} = 0.027$, $F_{neutralproteaseSS6/CTS+AA} = 7.685$, $p_{neutralproteaseSS6/CTS+AA} = 0.048$, $F_{neutralproteaseNS6/CTS+AA} = 11.803$, $p_{neutralproteaseNS6/CTS+AA} = 0.026$; $F_{acidproteaseSS6/control} = 59.442$, $p_{acidproteaseSS6/control} = 0.002$, $F_{acidproteaseNS6/control} = 121.931$, $p_{acidproteaseNS6/control} = 0.000$, $F_{acidproteaseSS6/AA} = 49.505$, $p_{acidproteaseSS6/AA} = 0.002$, $F_{acidproteaseNS6/AA} = 100.468$, $p_{acidproteaseNS6/AA} = 0.001$, $F_{acidproteaseSS6/CTS} = 46.324$, $p_{acidproteaseSS6/CTS} = 0.002$, $F_{acidproteaseNS6/CTS} = 97.105$, $p_{acidproteaseNS6/CTS} = 0.001$, $F_{acidproteaseSS6/CTS+AA} = 45.372$, $p_{acidproteaseSS6/CTS+AA} = 0.002$, $F_{acidproteaseNS6/CTS+AA} = 99.649$, $p_{acidproteaseNS6/CTS+AA} = 0.001$; $F_{\alpha-amylaseSS6/control} = 89.072$, $p_{\alpha-amylaseSS6/control} = 0.001$, $F_{\alpha-amylaseNS6/control} = 100.051$, $p_{\alpha-amylaseNS6/control} = 0.001$, $F_{\alpha-amylaseSS6/AA} = 76.470$, $p_{\alpha-amylaseSS6/AA} = 0.001$, $F_{\alpha-amylaseNS6/AA} = 87.134$, $p_{\alpha-amylaseNS6/AA} = 0.001$, $F_{\alpha-amylaseSS6/CTS} = 71.513$, $p_{\alpha-amylaseSS6/CTS} = 0.001$, $F_{\alpha-amylaseNS6/CTS} = 81.836$, $p_{\alpha-amylaseNS6/CTS} = 0.001$, $F_{\alpha-amylaseSS6/CTS+AA} = 68.208$, $p_{\alpha-amylaseSS6/CTS+AA} = 0.001$, $F_{\alpha-amylaseNS6/CTS+AA} = 78.214$, $p_{\alpha-amylaseNS6/CTS+AA} = 0.001$; $F_{cellulaseSS6/control} = 87.203$, $p_{cellulaseSS6/control} = 0.001$, $F_{cellulaseNS6/control} = 79.853$, $p_{cellulaseNS6/control} = 0.001$, $F_{cellulaseSS6/AA} = 69.334$, $p_{cellulaseSS6/AA} = 0.001$, $F_{cellulaseNS6/AA} = 65.471$, $p_{cellulaseNS6/AA} = 0.001$, $F_{cellulaseSS6/CTS} = 85.348$, $p_{cellulaseSS6/CTS} = 0.001$, $F_{cellulaseNS6/CTS} = 77.136$, $p_{cellulaseNS6/CTS} = 0.001$, $F_{cellulaseSS6/CTS+AA} = 70.773$, $p_{cellulaseSS6/CTS+AA} = 0.001$, $F_{cellulaseNS6/CTS+AA} = 65.305$, $p_{cellulaseNS6/CTS+AA} = 0.001$; $F_{glucoamylaseSS6/control} = 16.454$, $p_{glucoamylaseSS6/control} = 0.015$, $F_{glucoamylaseNS6/control} = 16.782$, $p_{glucoamylaseNS6/control} = 0.015$, $F_{glucoamylaseSS6/AA} = 16.202$, $p_{glucoamylaseSS6/AA} = 0.016$, $F_{glucoamylaseNS6/AA} = 16.508$, $p_{glucoamylaseNS6/AA} = 0.015$, $F_{glucoamylaseSS6/CTS} = 12.522$, $p_{glucoamylaseSS6/CTS} = 0.024$, $F_{glucoamylaseNS6/CTS} = 13.021$, $p_{glucoamylaseNS6/CTS} = 0.023$, $F_{glucoamylaseSS6/CTS+AA} = 9.776$, $p_{glucoamylaseSS6/CTS+AA} = 0.035$, $F_{glucoamylaseNS6/CTS+AA} = 10.389$, $p_{glucoamylaseNS6/CTS+AA} = 0.032$; $F_{pectinaseSS6/control} = 65.523$, $p_{pectinaseSS6/control} = 0.001$, $F_{pectinaseNS6/control} = 82.157$, $p_{pectinaseNS6/control} = 0.001$, $F_{pectinaseSS6/AA} = 70.167$, $p_{pectinaseSS6/AA} = 0.001$, $F_{pectinaseNS6/AA} = 88.994$, $p_{pectinaseNS6/AA} = 0.001$, $F_{pectinaseSS6/CTS} = 43.306$, $p_{pectinaseSS6/CTS} = 0.003$, $F_{pectinaseNS6/CTS} = 54.594$, $p_{pectinaseNS6/CTS} = 0.002$, $F_{pectinaseSS6/CTS+AA} = 40.891$, $p_{pectinaseSS6/CTS+AA} = 0.003$,

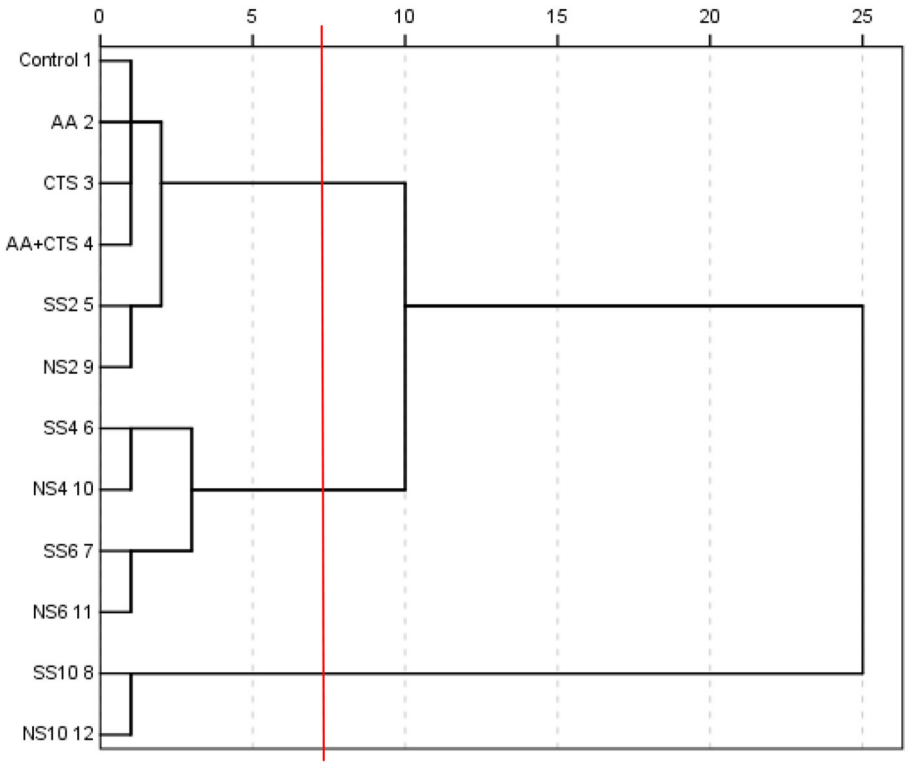

FIGURE 3
Result of cluster analysis of soy sauces.

$F_{pectinaseNS6/CTS+AA} = 51.624$, $p_{pectinaseNS6/CTS+AA} = 0.002$) and higher than those of SS2, SS4, SS10, NS2, NS4 and NS10 to some extent, but there was no marked differences in the neutral protease, acid protease, α-amylase, cellulase, glucoamylase and pectinase activities between SS6 and NS6 ($F_{neutralproteaseSS6/NS6} = 0.803$, $p_{neutralproteaseSS6/NS6} = 0.421$; $F_{acidproteaseSS6/NS6} = 1.799$, $p_{acidproteaseSS6/NS6} = 0.251$; $F_{\alpha-amylaseSS6/NS6} = 0.358$, $p_{\alpha-amylaseSS6/NS6} = 0.582$; $F_{cellulaseSS6/NS6} = 0.287$, $p_{cellulaseSS6/NS6} = 0.626$; $F_{glucoamylaseSS6/NS6} = 0.057$, $p_{glucoamylaseSS6/NS6} = 0.823$; $F_{pectinaseSS6/NS6} = 0.386$, $p_{pectinaseSS6/NS6} = 0.568$). The above analyses indicated the optimal concentration of nano-selenium used in soybean soaking was 6 mg/L. An appropriate concentration of selenium could effectively maintain the integrity of microbial cells and improve the activities of selenium dependent enzymes in microorganisms, so as to promote the growth of microorganisms and secrete more enzymes. However, once the concentration of selenium is excessively high (especially more than 10 mg/L), selenium exhibits toxicity, which leads to oxidative stress of microorganisms. A large number of reactive oxygen free radicals would destroy the metabolic enzyme system of microorganisms, resulting in the loss of protein activity, thus inhibiting the growth of microorganisms (33).

Proteases secreted by *Aspergillus oryzae*, especially acid proteases, could degrade proteins into peptides, oligopeptides and amino acids (36). Basically, the FAAs content in soy sauce was positively related to the activity of proteases in kojis. *A. oryzae* can also secrete cellulase and pectinase to degrade the soybean cell wall components, so that the total phenolics and total flavonoids attached to the soybean cell wall in the form of esterification or insoluble binding were released from the cell wall (16). Thus, the increased cellulase and pectinase activities could be of help to the release of total phenolics and total flavonoids from the soybean cell wall.

TABLE 7 Source of variation for the principal component (PC).

Item	First principal component (PC1)	Second principal component (PC2)
Total phenolics	0.996	−0.003
Total flavonoids	0.969	0.019
Formaldehyde nitrogen	0.991	0.020
Total titratable acid	0.991	−0.094
Reducing sugar	0.959	−0.192
Total sugar	0.981	−0.065
Maillard reaction products	0.967	−0.070
Non-salt soluble solids	0.992	−0.030
Total selenium	0.254	0.966
Inorganic selenium	0.112	0.993
Organic selenium	0.268	0.962
Free amino acids	0.987	−0.146
DPPH radical scavenging activity	0.996	−0.041
ABTS radical scavenging activity	0.977	−0.097
Reducing power	0.982	0.080
Metal ion chelating activity	0.992	−0.013

TABLE 6 Initial eigenvalue and cumulative variance contribution rate of principal components.

Principal component	Characteristic value	Variance contribution rate %	Cumulative variance contribution rate %
1	12.712	79.452	79.452
2	2.940	18.372	97.825
3	0.153	0.955	98.780
4	0.094	0.587	99.367
5	0.042	0.260	99.627
6	0.030	0.188	99.815
7	0.016	0.101	99.916
8	0.006	0.037	99.953
9	0.006	0.035	99.988
10	0.002	0.009	99.997
11	0.000	0.003	100.000
12	1.561E-16	9.753E-16	100.000
13	1.096E-16	6.849E-16	100.000
14	−8.718E-18	−5.449E-17	100.000
15	−7.695E-17	−4.809E-16	100.000
16	−3.770E-16	−2.356E-15	100.000

TABLE 8 Main component score and comprehensive score of soy sauces.

Number	Sample name	PC1 score	PC2 score	Comprehensive score	Comprehensive score ranking
1	Control	−3.24971	−1.25630	−2.87528	12
2	AA	−3.14403	−1.27655	−2.79325	11
3	CTS	−2.53603	−1.34253	−2.31185	10
4	AA+CTS	−2.25557	−1.37982	−2.09108	9
5	SS2	−1.05910	−0.70342	−0.99229	6
6	SS4	1.91351	−0.08098	1.53887	4
7	SS6	5.50058	0.62814	4.58536	2
8	SS10	−3.07821	3.82273	−1.78197	8
9	NS2	−0.24238	−0.90597	−0.36702	5
10	NS4	3.12278	−0.54891	2.43311	3
11	NS6	7.03630	0.07910	5.72949	1
12	NS10	−2.00810	2.96453	−1.07406	7

Combined with the above analyses, it could be concluded that appropriate content of selenium used during soybean soaking could promote the growth of *A. oryzae* and improve the activities of neutral protease, acid protease, α-amylase, cellulase, glucoamylase and pectinase, which were beneficial to increasing the contents of small peptides, free amino acids, Maillard reaction products, total phenolics and total flavonoids in soy sauces, so as to improve the antioxidant activities of soy sauce.

Cluster analysis

Four indices of antioxidant activities (DRSA, ARSA, RP and MICA) and 12 active ingredients (TP, TF, FN, TTA, RS, TS, MRP, NSS, TSe, ISe, OSe and FAAs) of soy sauces were systematically clustered according to the square euclidean distance, the results were shown in Figure 3.

When the distance was more than 5 and less than 10 (i.e., the red line in Figure 3), soy sauces could be well divided into three categories, among which NS6, SS6, NS4 and SS4 were the first category (C1), NS10 and SS10 were the second category (C2), and NS2, SS2, AA+CTS, CTS, AA and the control were the third category (C3). It can be concluded that group C1 had the strongest antioxidant activity, followed by group C2 and C3 according to the above analyses.

The results suggested that low concentration of nano-selenium and sodium selenite, ascorbic acid and chitosan contained in nano-selenium had no significant effects on the release and formation of various active ingredients and the antioxidant activities of soy sauces. Therefore, NS2, SS2, AA+CTS, CTS, AA and the control were classified into the same category (C3). The appropriate concentration of selenium could increase the contents of selenium and various active ingredients in soy sauces, so as to improve the antioxidant activities of soy sauces. Therefore, NS6, SS6, NS4 and SS4 were classified into the same category (C1). Interestingly, nano-selenium and sodium selenite with the same concentration in C1, C2 and C3 were all divided into the same smallest categories, indicating the insignificant effects of nano-selenium and sodium selenite with the same concentration on the antioxidant activities of soy sauces.

Principal component analysis

Principal component analysis was conducted on the original data of 16 variables including antioxidant activities (DRSA, ARSA, RP and MICA) and active ingredients (TP, TF, FN, TTA, RS, TS, MRP, NSS, TSe, ISe, OSe and FAAs). In order to ensure the integrity and reliability of information, the cumulative variance contribution rate should be over 80%, and the results were shown in Tables 6–8.

According to Table 6, the cumulative variance contribution rate was 97.83%, and the eigenvalues of the first two principal components were higher than 1, thus the first two principal components were extracted. Among them, the eigenvalue of the first principal component (PC1) was 12.71, which was the most important factor, explaining 79.45% of the variation. As shown in Table 7, FN, TTA, RS, TS, MRP, NSS, FAAs, TP, TF, DRSA, ARSA, RP and MICA had a high load on PC1, indicating that PC1 primarily represented the information of these indicators. The eigenvalue of the second principal component (PC2) was 2.94, PC2 explained 18.37% of the variation. As shown in Table 7, TSe, ISe and OSe had a high load on PC2, indicating that PC2 primarily represented the information of these indicators. Since the extraction of the first two principal components could primarily represent the information of all indicators, the two new variables (PC1 and PC2) were used to replace the original 16 variables. PC1 score, PC2 score and comprehensive score were calculated. As exhibited in Table 8, the results demonstrated that

the comprehensive score of NS6 ranked 1, followed by SS6, NS4, SS4, NS2, SS2, NS10, SS10, AA+CTS, CTS, AA and the control.

The above principal component analysis confirmed again that the appropriate concentration of selenium not only increased the contents of total selenium and organic selenium in soy sauce, but also enhanced the soy sauce's antioxidant activities.

Conclusion

In light of the above analyses, compared with the control, the appropriate concentration of selenium (6 mg/L nano-selenium) could increase the contents of total selenium and organic selenium in soy sauce significantly, promote the growth and metabolism of *A. oryzae*, and make the latter secrete more proteases, amylases and other enzymes. The enzymes were beneficial to promoting the release and formation of antioxidant compounds including phenolics, flavonoids, free amino acids and Maillard reaction products during moromi fermentation, and the latter enhanced the antioxidant activities of soy sauce. In addition, in view of nano-selenium had lower toxicity and higher biological activities than sodium selenite, nano-selenium may have a broader application prospect in selenium-enriched soy sauce industry in the future. In conclusion, the work developed a widely accessible selenium supplement and a novel method to enhance organic selenium content and antioxidant activities of soy sauce and clarified the related mechanisms. Further work on the effects of nano-selenium on aroma and taste of soy sauce and the purification and identification of selenium-containing peptides with strong antioxidant activities in soy sauce is in progress.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JC and TF involved in investigation, data curation, formal analysis, and writing original draft. BW contributed to conceptualization, visualization, resources, and writing review and editing. RH performed methodology, supervision, validation, and writing review and editing. YX and PG involved in investigation and formal analysis. Z-HZ and LZ contributed to conceptualization, methodology, resources, and visualization. JF and ZL involved in conceptualization, formal analysis, validation, and resources. XG contributed to conceptualization, project administration, methodology, resources, supervision, and writing review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors JF and ZL were employed by Guangdong Chubang Food Co., Yangjiang, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Lentinan-functionalized selenium nanoparticles induce apoptosis and cell cycle arrest in human colon carcinoma HCT-116 cells

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Selenium nanoparticles (SeNPs) have gained extensive attention for their excellent biological activity and low toxicity. However, SeNPs are extremely liable to aggregate into non-bioactive or gray elemental selenium, which limits their application in the biomedicine field. This study aimed to prepare stable SeNPs by using lentinan (LNT) as a template and evaluate its anti-colon cancer activity. The average particle diameter of obtained lentinan-selenium nanoparticles (LNT-SeNPs) was approximately 59 nm and presented zero-valent, amorphous, and spherical structures. The monodisperse SeNPs were stabilized by LNT through hydrogen bonding interactions. LNT-SeNPs solution remained highly stable at 4°C for at least 8 weeks. The stability of LNT-SeNPs solution sharply decreased under high temperature and strong acidic conditions. LNT-SeNPs showed no obvious cytotoxic effect on normal cells (IEC-6) but significantly inhibited the proliferation of five colon cancer cells (HCT-116, HT-29, Caco-2, SW620, and CT26). Among them, LNT-SeNPs exhibited the highest sensitivity toward HCT-116 cells with an IC_{50} value of 7.65 μM. Also, LNT-SeNPs displayed better cancer cell selectivity than sodium selenite and selenomethionine. Moreover, LNT-SeNPs promoted apoptosis of HCT-116 cells through activating mitochondria-mediated apoptotic pathway. Meanwhile, LNT-SeNPs induced cell cycle arrest at G0/G1 phase in HCT-116 cells *via* modulation of cell cycle regulatory proteins. The results of this study indicated that LNT-SeNPs possessed strong potential application in the treatment of colorectal cancer (CRC).

KEYWORDS

lentinan, selenium nanoparticles, characterization, stability, HCT-116, apoptosis, cell cycle arrest

Introduction

Colorectal cancer (CRC) is one of the most serious and formidable malignancies, with more than 1.88 million (excluding anus) new cases and 915,000 deaths estimated to occur in 2020. Overall, CRC is the third most frequently occurring cancer and the second leading cause of cancer death worldwide (1). In 2020, the incidence and mortality rates of CRC in China increased to 28.8 and 30.6%, respectively (2). Except for genetic factors, about 50% of CRC are estimated to be influenced by dietary factors (3). In recent decades, dietary chemopreventive agents have drawn much attention and may be an effective approach for impeding or delaying the development of CRC (4).

Selenium (Se), an essential trace element, plays an important role in balancing redox systems and regulating immune function (5). Se as a potential chemopreventive agent is gaining considerable attention. Se compounds have been reported to reduce the risk of several cancer types including colorectal, lung, breast, bladder, and prostate cancers (6). Nevertheless, Se displays a very narrow margin between beneficial dose and toxic dose. A high level of Se intake for susceptible patients can result in some symptoms of selenosis (7). Various studies have demonstrated that the chemical forms of Se compounds are closely related to their beneficial and toxic effects (8, 9). Compared with inorganic and organic Se compounds, Selenium nanoparticles (SeNPs) present in the form of zero-valent status exhibit better bioavailability, lower toxicity, and stronger bioactivity (10, 11). However, SeNPs synthesized by redox reactions are usually unstable and liable to aggregate, resulting in a great reduction of bioactivity and bioavailability (5, 12). Therefore, massive efforts have been taken to stabilize SeNPs through the introduction of templates, which can interact with SeNPs and suppress their aggregation. Besides, using bioactive templates will be helpful for regulating or enhancing the bioactivities of SeNPs (13).

Polysaccharides have a wide range of biological activities such as anti-tumor, antioxidation, and immunoregulation (14). Numerous studies have confirmed that various polysaccharides can be used as bioactive templates to stabilize SeNPs. Recent studies showed that SeNPs stabilized by polysaccharides from *Pleurotus tuber-regium*, *Ganoderma lucidum* (15), *Polyporus umbellatus* (10), and *Oudemansiella raphanipies* (16) possessed anti-tumor efficacy. Lentinan (LNT), a natural β-1,3-glucan polysaccharide isolated from *Lentinus edodes*, has been used for adjuvant tumor therapy in Japan and China (17). During 2004–2016, over 17.4% of LNT treatments were given to patients with CRC in China (18). LNT has been regarded as a potential adjuvant for CRC therapy because LNT combined with chemotherapeutics can improve survival rates, enhance immune function, and reduce side effects (19). Jia et al. (20) reported that SeNPs decorated with single-chain LNT (s-LNT) inhibited the proliferation of Hela cells. Liu et al. (21) showed that s-LNT-functionalized SeNPs could effectively inhibit malignant ascites *via* the toll-like receptor 4/TNF receptor-associated factor 3/mitofusin 1 pathway.

However, to our knowledge, the anti-colon cancer activity of lentinan-selenium nanoparticles (LNT-SeNPs) remains virtually unknown. Also, the physicochemical characterization and stability of LNT-SeNPs have not been well-documented. In this study, we synthesized LNT-SeNPs in the redox system and examined its size, morphology, crystal form, elemental composition, valence state, and binding mechanism. Moreover, the stability test was carried out under different temperature, time, and pH conditions. In addition, five colon cancer cell lines (HCT-116, HT-29, Caco-2, SW620, and CT26) combined with a normal small intestine epithelium cell line (IEC-6) were used to evaluate the cytotoxic effects of LNT-SeNPs. Finally, the apoptosis induction and cell cycle arrest of LNT-SeNPs in HCT-116 cells and its underlying molecular mechanisms were further investigated.

Materials and methods

Materials and chemicals

LNT and selenomethionine (SeMet) were obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Sodium selenite (Na_2SeO_3) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, United States). Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, RIPA buffer, Pierce BCA protein assay kit, eBioscience™ Annexin V apoptosis detection kit, and NuPAGE Bis-Tris gels were obtained from Thermo Scientific (Rockford, United States). Mitochondrial membrane potential (MMP) assay kit and cell cycle analysis kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Caspase-9 and −3 colorimetric assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The cytochrome c, Bcl-2, Bax, β-actin, p21, p27, CDK2, and CDK4 antibodies were obtained from Cell Signaling Technology (Boston, United States). The cyclin D1 and CDK6 antibodies were obtained from Abcam (Cambridge, United States).

Preparation of lentinan-selenium nanoparticles

LNT-SeNPs were prepared according to a previous method (10). Briefly, LNT solution (0–8 mL, 2.5 mg/mL) were mixed with Na_2SeO_3 solution (1 mL, 50 mM) under magnetic stirring for 5 min. Then ascorbic acid solution (10 mL, 20 mM) was dropwise added into the mixture. After reconstituting to 20 mL with ultrapure water, the system was stirred for 4 h at 40°C in the dark. Finally, excessive ascorbic acid and Na_2SeO_3 were removed by dialysis for 72 h at 4°C.

Characterization of lentinan-selenium nanoparticles

The concentration of Se was determined by a NexION 350 inductively coupled plasma-mass spectroscopy (ICP-MS, PerkinElmer, United States). The average particle size was measured by a Zetasizer Nano ZS90 particle analyzer (Malvern, United Kingdom). The transmission electron microscopy (TEM) images were carried out with an H-7650 (Hitachi, Japan). The high-resolution TEM (HRTEM) images and corresponding selected area electron diffraction (SAED) pattern were taken on a Talos F200X (FEI, United States) operated at 200 kV. The crystal form was analyzed on a D8 Advance X-ray diffractometer (Bruker, Germany) operated at 40 kV and 40 mA. The patterns were recorded from 5° to 90° at a speed of 10°/min. The valence state was analyzed by an Axis Ultra DLD X-ray photoelectron spectrometer (XPS, Kratos, United Kingdom) equipped with a monochromatic Al Kα X-ray source. The spectra were calibrated with C 1s (284.6 eV). The ultraviolet and visible (UV–Vis) spectra were measured using a U-2910 spectrophotometer (Hitachi, Japan). The Fourier transform infrared spectroscopy (FT-IR) spectra were carried out on a Vertex 70 spectrometer (Bruker, Germany) at the range of 4,000–400 cm^{-1} using the KBr disk method.

Stability of lentinan-selenium nanoparticles

The stability experiment was explored under different temperature, time, and pH. For temperature, the average particle diameters of LNT-SeNPs were determined after 3 days of storage at 4, 25, 37, and 60°C, respectively. During the 16 weeks of storage at 4°C, the mean diameters of LNT-SeNPs solution were recorded after the 1st, 2nd, 4th, 6th, 8th, 12th, and 16th weeks. For pH levels, the mean diameters of LNT-SeNPs solution were detected after the pH value was adjusted to 2, 4, 6, 7.2, 8, 10, and 12 by HCl or NaOH. The effects of pH value at simulative gastrointestinal environments on the stability of LNT-SeNPs were further evaluated. Firstly, the pH of LNT-SeNPs solution was adjusted to 2 by HCl to mimic gastric environment. The mean diameter was recorded after 1 h incubation at 37°C. Subsequently, the pH of LNT-SeNPs solution was adjusted from 2 to 7.2 by NaOH to simulate intestinal environment. The mean diameter was measured after 2 h incubation at 37°C.

Cell culture

Human colon cancer cell lines (HCT-116, HT-29, Caco-2, and SW620), mouse colon cancer cell line (CT26), and rat small intestine epithelium cell line (IEC-6) were purchased from the Chinese Academy of Sciences (Shanghai, China). HT-29 and SW620 cells were cultured in a humidified incubator at 37°C with 5% CO_2 in DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 U/mL). The other cells were maintained in RPMI 1640 medium.

Cell viability assay

Cell viability was monitored by the MTT reduction assay (22). Cells (HCT-116, HT-29, Caco-2, SW620, CT26, and IEC-6) were cultivated at a density of 5×10^4 cells/mL on a 96-well plate for 24 h. Then cells were treated with various concentrations (2, 4, 8, 16, 32, and 64 μM) of LNT-SeNPs for another 48 h. Subsequently, the supernatant was removed carefully and 100 μL of MTT (0.25 mg/mL) solution was added into each well. After 2 h incubation, the dark blue formazan crystals were dissolved with 200 μL of dimethyl sulfoxide under gentle shaking. The absorbance at 550 nm was measured with a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, United States). Cell viability was determined relative to the control group cultured with medium only (100%). To compare the cytotoxic effects of LNT-SeNPs, Na_2SeO_3, and SeMet on HCT-116 and IEC-6 cells, the cell viability was conducted as mentioned above.

Apoptosis assay

Cell apoptosis was measured by the Annexin V apoptosis detection kit according to the manufacturer's protocols. After treatment with LNT-SeNPs (3, 6, and 12 μM) for 48 h, HCT-116 cells were harvested and washed with DPBS. Then, the cells were incubated with Annexin V-fluorescein isothiocyanate and propidium iodide (PI) at room temperature in the dark. Stained cells were measured by a CytoFLEX flow cytometer (Beckman Coulter, Brea, United States). Data analysis was performed by CytExpert 2.0 software (Beckman Coulter).

Determination of mitochondrial membrane potential

The MMP was detected using JC-1 fluorescence probe. After treatment with LNT-SeNPs (3, 6, and 12 μM) for 48 h, HCT-116 cells were collected and incubated with JC-1 based on the manufacturer's protocols. Stained cells were measured using Beckman flow cytometer. Data analysis was performed by CytExpert 2.0 software (Beckman Coulter).

Measurement of caspase activity

HCT-116 cells were treated with various concentrations (3, 6, and 12 μM) of LNT-SeNPs for 48 h, and then

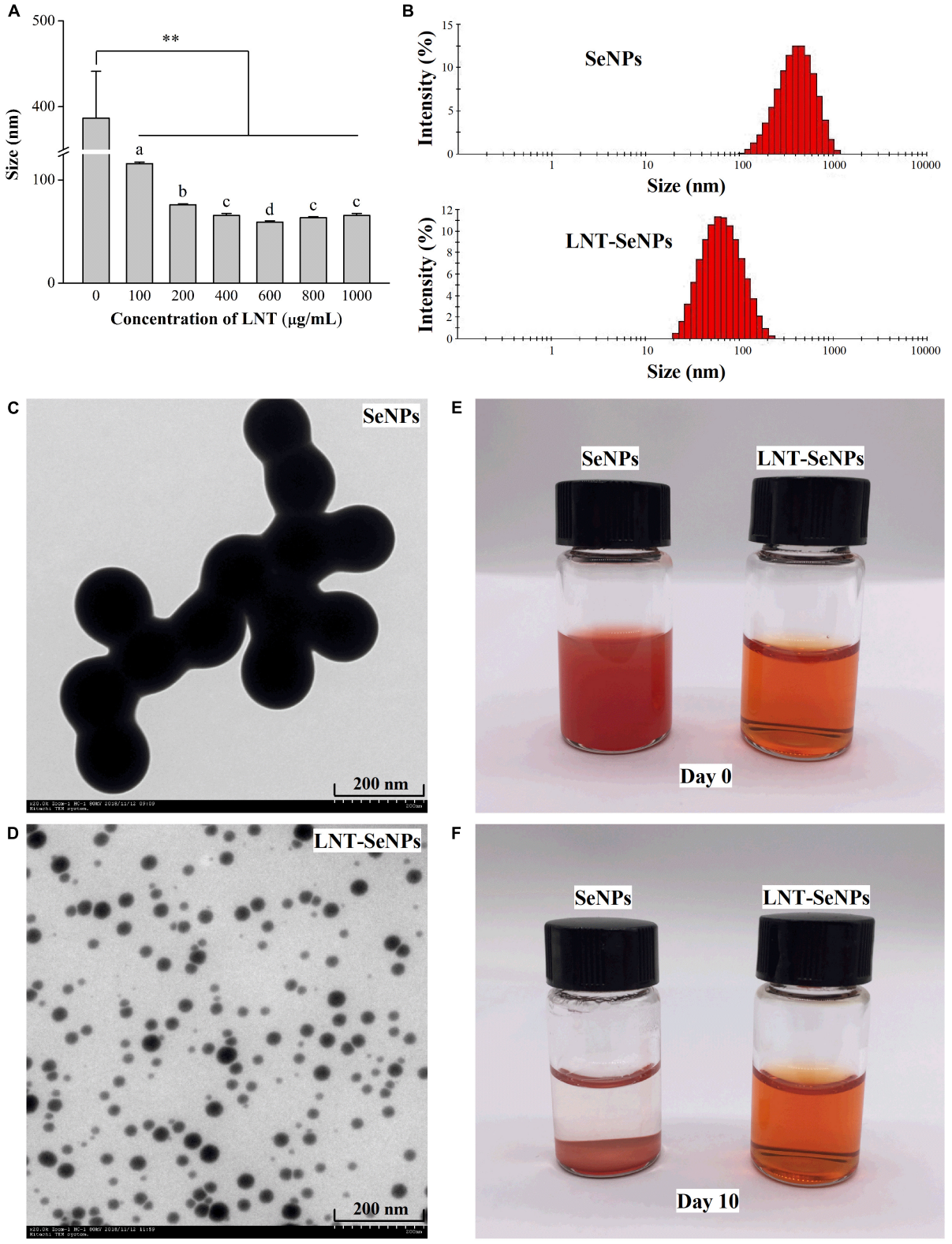

FIGURE 1
The morphology feature and particle size of SeNPs and LNT-SeNPs. Average particle diameter of SeNPs prepared at different concentrations of LNT **(A)**; size distribution of SeNPs and LNT-SeNPs **(B)**; TEM image of SeNPs **(C)** and LNT-SeNPs **(D)**; photographs of SeNPs and LNT-SeNPs at day 0 **(E)** and day 10 **(F)**. $^{**}p < 0.01$ represents significant difference between SeNPs alone and SeNPs with LNT as a template. Different letters represent significant difference ($p < 0.05$).

lysed to obtain the cell lysate. The caspase-9 and −3 activities were measured based on the manufacturer's instructions.

Cell cycle assay

The distribution of cell cycle was detected using the cell cycle analysis kit. After treatment with LNT-SeNPs (3, 6, and 12 μM) for 48 h, HCT-116 cells were collected and fixed with precooled 70% ethanol overnight at 4°C. Prior to analysis, cells were washed with DPBS and incubated with PI and RNase A for 0.5 h at room temperature. Stained cells were measured by Beckman flow cytometer. The cell cycle analysis was performed by Flowjo 7.6 software (Tree Star Inc.).

Western blot analysis

HCT-116 cells were incubated with 3, 6, and 12 μM of LNT-SeNPs for 48 h. Then cells were lysed by RIPA buffer supplemented with protease inhibitors. Protein concentrations were measured by the BCA method. Subsequently, equal amounts of protein were separated by NuPAGE Bis-Tris gels and transferred onto PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated at 4°C overnight with specific primary antibodies (Bcl-2, Bax, cytochrome c, p21, p27, cyclin D1, CDK2, CDK4, CDK6, and β-actin. Then the membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies. Finally, the protein bands were detected with enhanced chemiluminescence reagent using an Omega Lum G imaging system. The relative density of protein bands was normalized to β -actin.

Statistical analysis

Experimental results were presented as means ± standard deviation (*SD*). Significant differences were analyzed by ANOVA and Duncan's multiple-range test using SAS 9.2 software (Cary, United States). Data graphs were drawn by Origin 9.0 software (Northampton, United States).

Results and discussion

Morphology and physicochemical characterization of lentinan-selenium nanoparticles

In this study, synthesized SeNPs were decorated by using LNT as a template. As presented in **Figure 1A**, LNT at the concentration range of 100–1,000 μg/mL significantly decreased the average particle diameter of SeNPs. LNT at 600 μg/mL optimally dropped the mean size from 386.8 to 59.2 nm and the particle size distribution from 105.7–1106.0 to 21.0–220.2 nm (**Figure 1B**). The Se concentration of LNT-SeNPs solution was about 1.69 mM and the Se content accounted for 18.2%. TEM photographs showed that LNT-SeNPs were monodisperse spherical particles in the solution, while SeNPs with no addition of LNT tended to aggregate and adhere to each other (**Figures 1C,D**). The particle sizes of LNT-SeNPs and SeNPs observed under the TEM supported the above results of particle analyzer. Additionally, a transparent orange-red solution was obtained in the presence of LNT as a template during the preparation of SeNPs. After storage for 10 days, the LNT-SeNPs solution remained transparent but SeNPs alone appeared obvious precipitation (**Figures 1E,F**). These findings indicated that the introduction of LNT played a crucial role in the stability of SeNPs.

The crystal structure of LNT-SeNPs was performed by HRTEM and XRD. As shown in **Figure 2A**, no lattice fringe and SAED pattern of concentric rings were observed in the HRTEM. Moreover, XRD patterns showed that LNT-SeNPs had broad amorphous bands at lower angles (**Figure 2B**). These results suggested the amorphous nature of LNT-SeNPs. Consistent with our results, recent studies reported that the amorphous structure of SeNPs stabilized by glucan or chitosan was observed in the XRD patterns (13, 23). The valence state of Se in LNT-SeNPs was analyzed by XPS. An obvious Se 3d peak was found in the XPS spectrum of LNT-SeNPs (**Figure 2C**). After deconvolution, three Se $3d_{5/2}$ peaks at 56.6, 56.0, and 55.7 eV were detected (**Figure 2D**), suggesting the zero-valent Se in LNT-SeNPs. These results confirmed that the amorphous and zero-valent state of LNT-SeNPs were successfully constructed. Several studies pointed out that the amorphous form and zero-valent state of SeNPs were closely related to their excellent bioactivity and low toxicity (23, 24). The UV–Vis and FT-IR spectroscopy were further conducted to confirm the interaction between LNT and SeNPs. As shown in **Figure 2E**, SeNPs alone exhibited strong absorption bands from 250 and 600 nm while LNT showed weak absorption from 250 to 800 nm. Obviously, the absorption peak of LNT-SeNPs was around 250 nm, which was different from that of LNT and SeNPs. FT-IR spectra of LNT, LNT-SeNPs, and SeNPs were shown in **Figure 2F**. The adsorption peak of LNT at 3369.3 cm^{-1} was assigned to the –OH stretching vibrations (25), which was shifted to 3325.1 cm^{-1} for LNT-SeNPs, suggesting the strong bonding interaction between hydroxyl groups of LNT and SeNPs. No obvious shifts of other absorption peaks were observed. These results implied that the association of LNT and SeNPs was probably attributed to hydrogen bonding interactions. Similar phenomena were found in previous documents that SeNPs could bind to the hydroxyl groups of polysaccharides from *Polygonatum sibiricum* (26), *Astragalus membranaceus* (27), and black fungus (28).

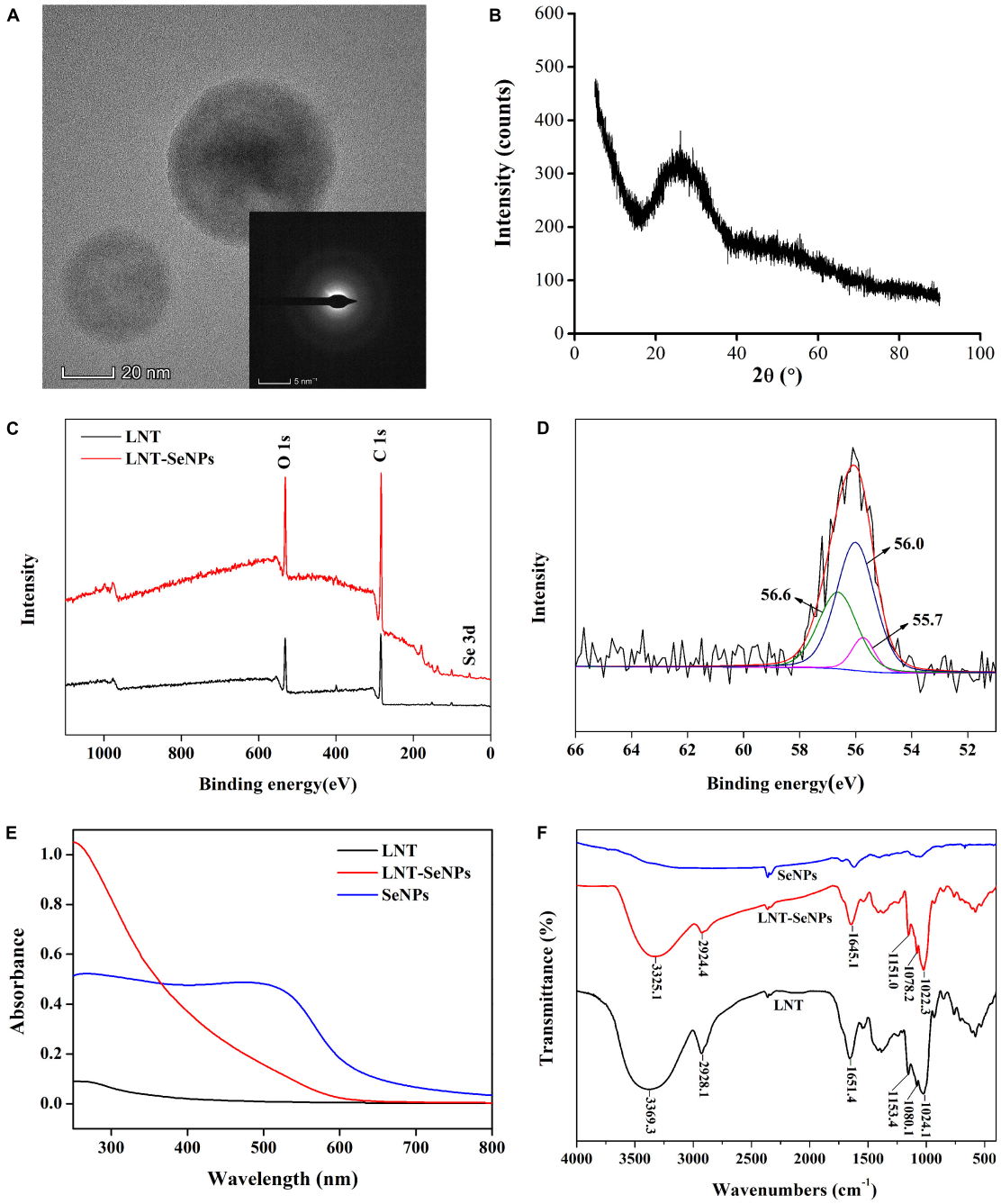

FIGURE 2
The physicochemical characterization of LNT-SeNPs. HRTEM image of LNT-SeNPs and its corresponding SAED pattern **(A)**; XRD pattern of LNT-SeNPs **(B)**; wide-range XPS patterns of LNT and LNT-SeNPs **(C)**; Se 3d spectra of LNT-SeNPs **(D)**; UV–Vis spectra of LNT, LNT-SeNPs and SeNPs **(E)**; FT-IR spectra of LNT, LNT-SeNPs and SeNPs **(F)**.

The stability of lentinan-selenium nanoparticles affected by temperature, time, and pH

The stability of LNT-SeNPs is crucial for its extensive application. Herein, we explored the effects of three key factors on the stability of LNT-SeNPs. It could be seen from **Figure 3A** that the stability of LNT-SeNPs markedly decreased with the rise of storage temperature (4–60°C) after 3 days of storage. Compared with storage at 4°C (58.8 nm), the particle diameter of LNT-SeNPs significantly increased to 63.6, 99.2, and 133.9 nm at 25, 37, and 60°C, respectively. Song et al. (23) reported that SeNPs stabilized by chitosan appeared aggregation and its

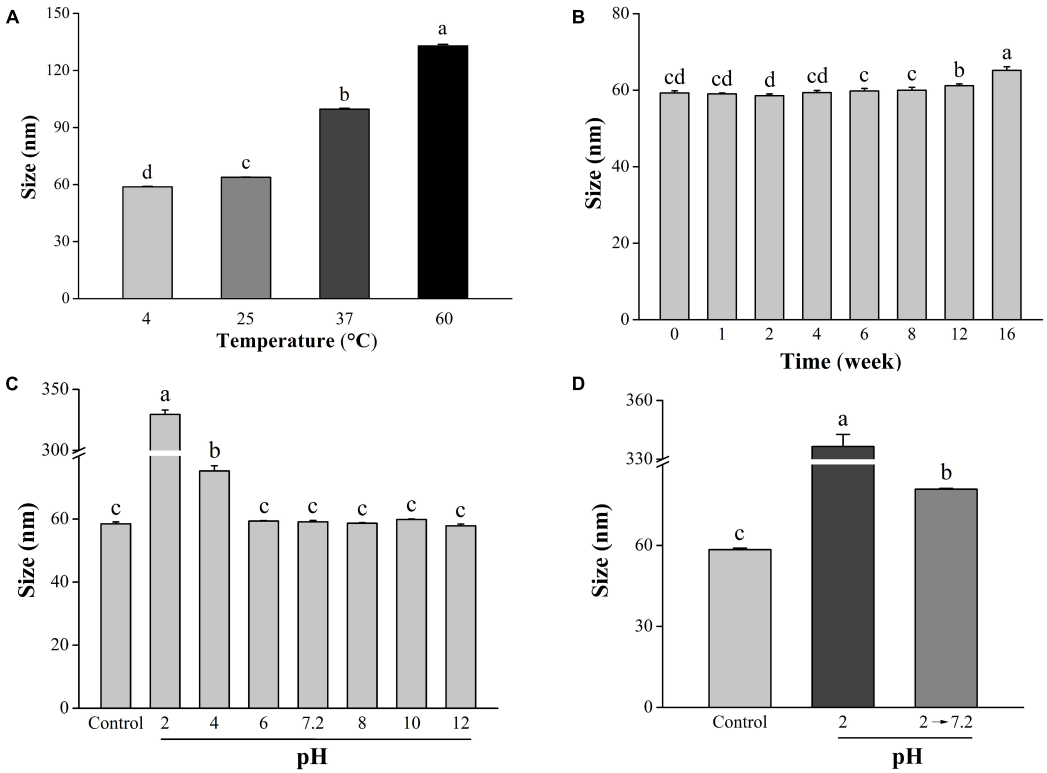

FIGURE 3
Effects of temperature, storage time, and pH on the stability of LNT-SeNPs. Changes in the average particle diameter of LNT-SeNPs placed in 4, 25, 37, and 60°C for 3 days **(A)**. Changes in the average particle diameter of LNT-SeNPs stored at 4°C for 0–16 weeks **(B)**; effect of pH on the average particle diameter of LNT-SeNPs **(C)**. Effect of pH at simulative gastrointestinal environments on the average particle diameter of LNT-SeNPs **(D)**. Data were shown as mean values ± *SD* (n = 3). Different letters represented significant differences ($p < 0.05$).

particle size significantly increased at 70°C, which is consistent with our results. As shown in Figure 3B, the particle size of LNT-SeNPs only slightly increased to 65.2 nm for 16 weeks of storage at 4°C. No obvious change in the particle diameter was observed at the 8th week, which is comparable to that of SeNPs stabilized by *Ulva lactuca* polysaccharide (29). As illustrated in Figure 3C, there was no significant change in the particle diameter of LNT-SeNPs at the pH range of 6–12. Nevertheless, the particle sizes remarkedly increased to 79.2 and 329.5 nm at pH 4 and 2, respectively. Interestingly, the particle size of LNT-SeNPs significantly decreased to 80.8 nm when the pH value was adjusted from 2 to 7.2 (Figure 3D). This phenomenon might be probably attributed to the protonation of LNT under strong acidic condition, which could attenuate the electrostatic interaction between LNT and SeNPs (30).

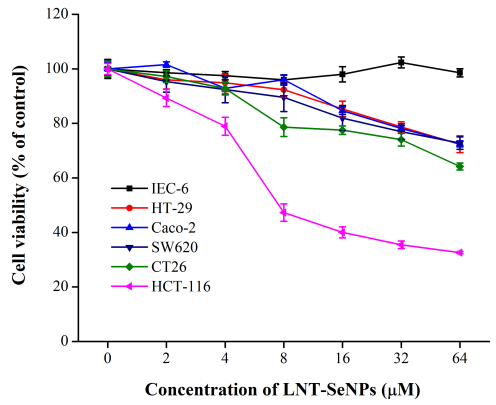

FIGURE 4
The cytotoxicity of LNT-SeNPs in colon cancer (HCT-116, HT-29, Caco-2, SW620, and CT26) and normal cells (IEC-6) at the concentration range of 2–64 μM. Data were shown as mean values ± *SD* (n = 6).

Cytotoxic effects of lentinan-selenium nanoparticles on colon cancer cells and normal cells

The anti- CRC activity of LNT-SeNPs was screened on HCT-116, HT-29, Caco-2, SW620, CT26, and IEC-6 cells using the MTT method. As shown in Figure 4, LNT-SeNPs displayed obvious anti-proliferative effects on all five cancer

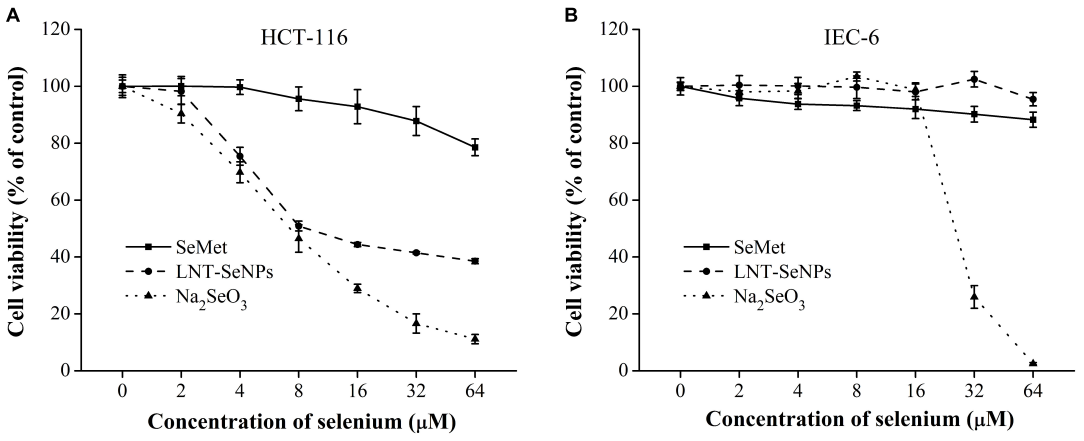

FIGURE 5
Cytotoxic effects of LNT-SeNPs, Na_2SeO_3, and SeMet on HCT-116 **(A)** and IEC-6 cells **(B)** at the concentration range of 2–64 μM. Data were shown as mean values ± *SD* (*n* = 6).

cells (HCT-116, HT-29, Caco-2, SW620, and CT26). In contrast, LNT-SeNPs exhibited no significant cytotoxicity toward the normal IEC-6 cells. This specific advantage of LNT-SeNPs might be conducive to reducing its side effects on normal tissues of patients during treatment. A similar result was found by Hu et al. (31) that SeNPs decorated with inulin fructan from *Codonopsis pilosula* had selectivity between cancer and normal cells. Compared with other cancer cells, LNT-SeNPs showed the strongest inhibitory effect on HCT-116 cells with an IC_{50} value of 7.65 μM. Therefore, HCT-116 cells were chosen as a cell model to explore the underlying mechanisms involved in the anti-proliferation of LNT-SeNPs in the subsequent experiment. Moreover, we compared the cytotoxic effect of LNT-SeNPs with Na_2SeO_3 and SeMet on HCT-116 and IEC-6 cells. As shown in **Figure 5A**, LNT-SeNPs and Na_2SeO_3 exhibited a better anti-proliferative effect on HCT-116 cells than SeMet. It needed to point out that Na_2SeO_3 possessed the strongest anti-proliferation at the Se concentration range of 16–64 μM. However, for normal IEC-6 cells, Na_2SeO_3 at 32 and 64 μM also strongly decreased cell viability to 30.0 and 2.5%, respectively. By contrast, the cell viabilities of LNT-SeNPs and SeMet at 64 μM were up to 95.5 and 88.3%, respectively (**Figure 5B**). These results implied that LNT-SeNPs exhibited better cancer cell selectivity than Na_2SeO_3 and SeMet. Consistent findings were also described in previous studies on the anti-proliferation of SeNPs decorated with chitosan (9) and *Polyporus umbellatus* polysaccharide (10).

Effect of lentinan-selenium nanoparticles on apoptosis in HCT-116 cells

The induction of apoptosis in cancer cells is a promising approach for cancer treatment (32). In this study, the pro-apoptotic effect of LNT-SeNPs on HCT-116 cells was further evaluated by Annexin V/PI double staining method using flow cytometry. As shown in **Figure 6A**, compared with the control group (2.6%), LNT-SeNPs treatment (3, 6, and 12 μM) significantly increased the total apoptotic percentage of HCT-116 cells to 4.8, 16.7, and 22.0%, respectively. These results indicated that LNT-SeNPs could exert apoptosis induction on HCT-116 cells, which might be partly associated with its anti-proliferation. It has been reported that mitochondria dysfunction can trigger the apoptosis of various cancer cells (33, 34). To explore whether the mitochondria dysfunction was involved in the apoptosis induction of LNT-SeNPs on HCT-116 cells, we determined the change of MMP *via* JC-1 staining assay. As presented in **Figure 6B**, the green fluorescence in right lower quadrant represented the loss of MMP in HCT-116 cells induced by LNT-SeNPs (34). Compared with the control group, LNT-SeNPs treatment (3, 6, and 12 μM) significantly and dose-dependently induced the loss of MMP in HCT-116 cells, indicating the occurrence of mitochondria dysfunction. The Bcl-2 family members consist of anti-apoptotic (such as Bcl-2) and pro-apoptotic (such as Bax) proteins (35). The up-regulation of Bax/Bcl-2 ratio can increase mitochondrial membrane permeability and the release of cytochrome c. Subsequently, caspase-9 is activated by the released cytochrome c, and then causes the activation of downstream caspase-3, resulting in apoptotic death (36). Our results showed that LNT-SeNPs treatment increased the expression of Bax and cytochrome c and decreased the level of Bcl-2 (**Figure 6C**). Additionally, LNT-SeNPs significantly induced a dose-dependent increase in the activities of caspase-9 and –3 in comparison to the control group (**Figure 6D**). These results indicated that LNT-SeNPs could induce apoptosis of HCT-116 cells *via* mitochondria-mediated intrinsic pathway.

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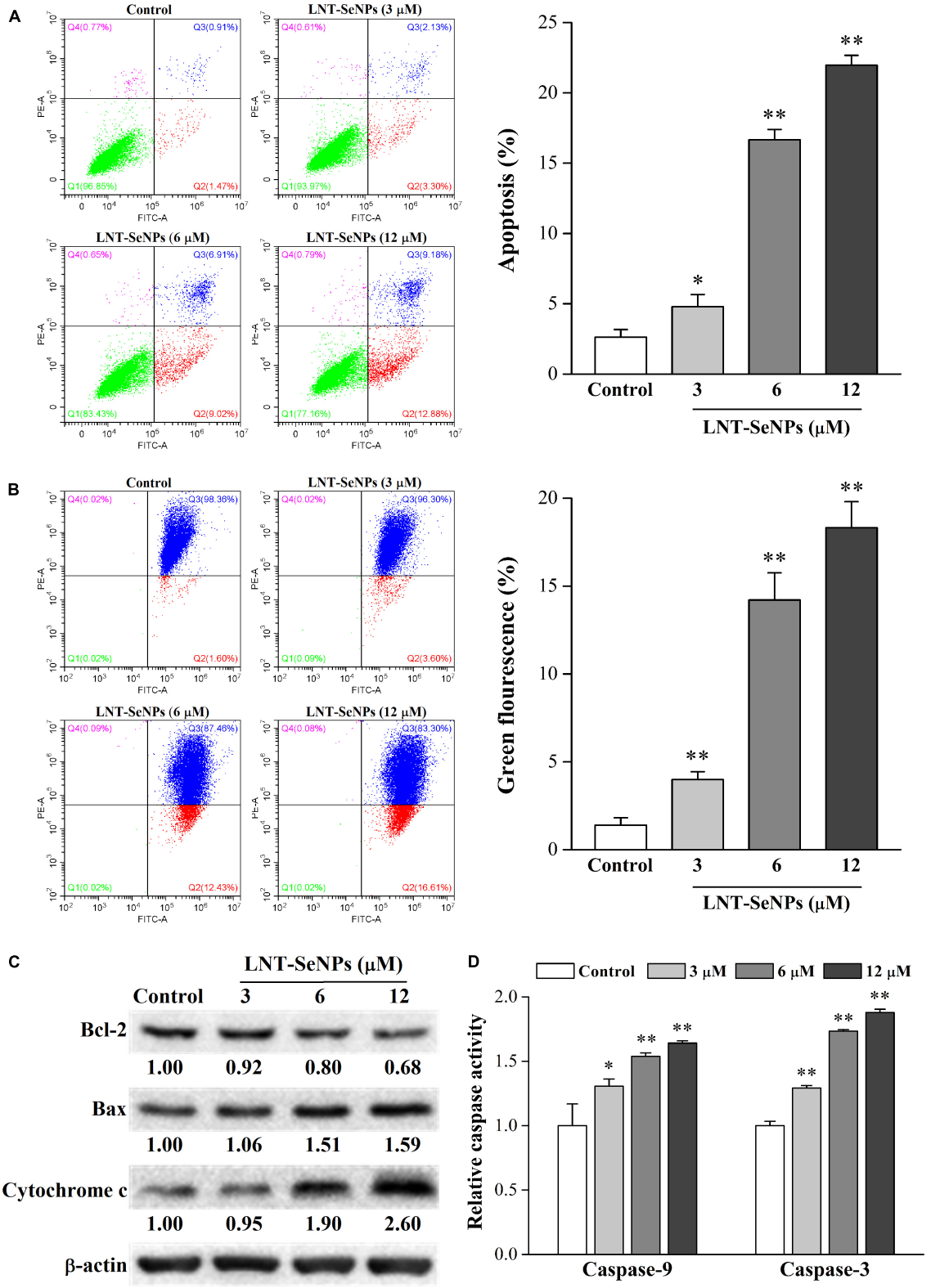

FIGURE 6
Effects of LNT-SeNPs treatment (3, 6, and 12 μM) on the mitochondria-mediated apoptotic pathway in HCT-116 cells. The apoptosis induction of HCT-116 cells was evaluated by Annexin V/PI double staining **(A)**; the change of MMP was measured by JC-1 staining **(B)**; the protein expression levels of Bcl-2, Bax, and cytochrome c were detected by western blot **(C)**; the caspase-9 and −3 activities were determined by colorimetric assay kit **(D)**. Data were shown as mean values ± *SD* (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the control group.

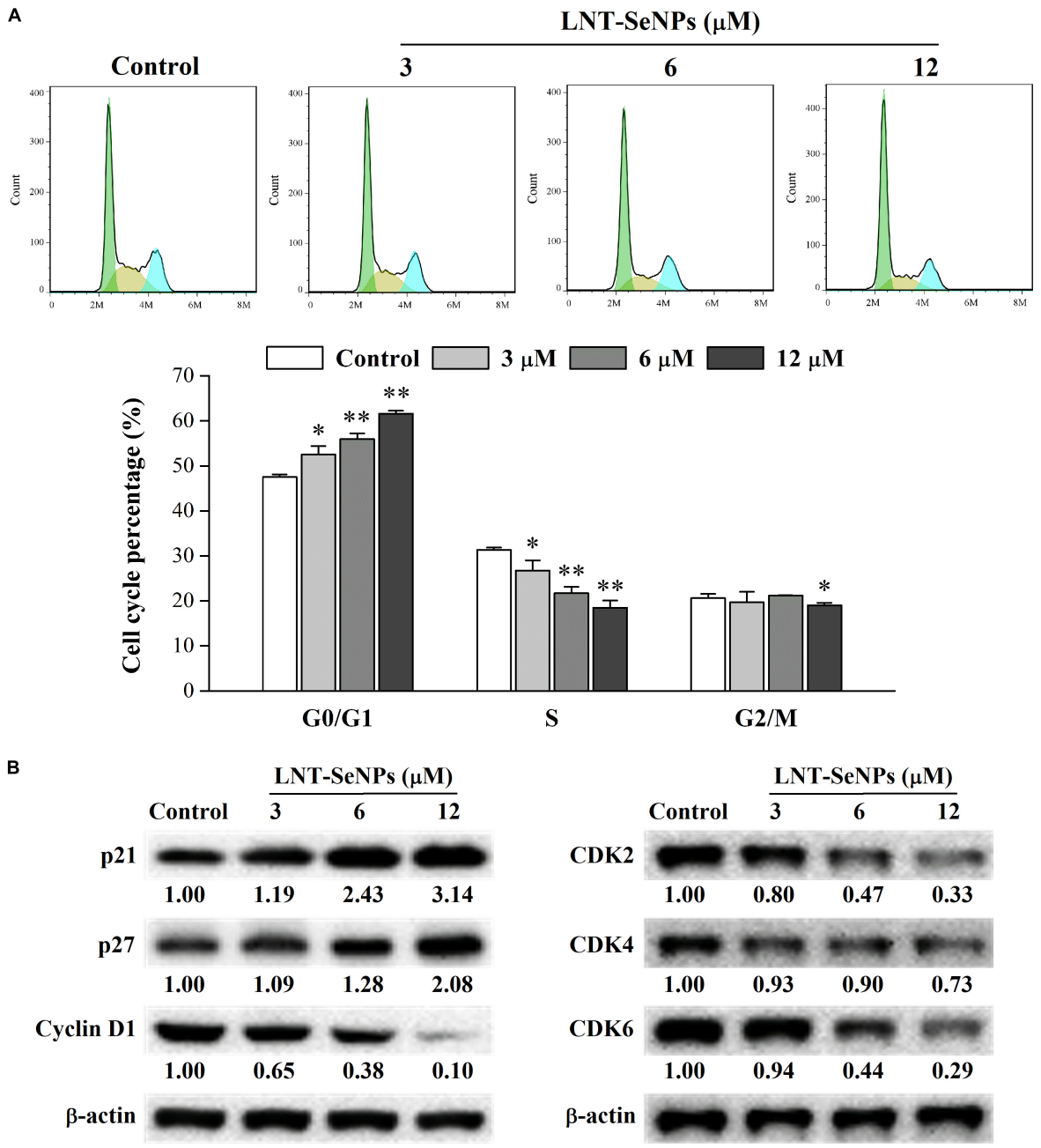

FIGURE 7
Effects of LNT-SeNPs treatment (3, 6, and 12 μM) on the cell cycle progression in HCT-116 cells. The cell cycle distribution of HCT-116 cells was analyzed by the PI staining method **(A)**; the protein expression levels of p21, p27, cyclin D1, CDK2, CDK4, and CDK6 were detected by western blot **(B)**. Data were shown as mean values ± *SD* (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the control group.

Effect of lentinan-selenium nanoparticles on cell cycle arrest in HCT-116 cells

Apart from apoptosis, cell cycle arrest is another crucial approach for preventing the proliferation of cancer cells (37). Thus, the cell cycle distribution of HCT-116 cells affected by LNT-SeNPs was further explored using flow cytometry. As presented in Figure 7A, LNT-SeNPs treatment significantly increased cell population in the G0/G1 phase in a dose-dependent manner compared with the control group. LNT-SeNPs at 12 μM induced G0/G1 phase arrest from 47.5 to 61.6% in HCT-116 cells. As a result of G0/G1 phase arrest, the proportions of cells in S and G2/M phases were remarkedly decreased from 31.3 to 18.5% and from 20.6 to

19.0%, respectively. These results suggested that the anti-proliferative activity of LNT-SeNPs in HCT-116 cells might be partly caused by G0/G1 phase cell arrest. However, Huang et al. (38) reported that *Pleurotus tuber-regium*-conjugated SeNPs induced G2/M phase arrest in HCT-116 cells. The inconsistent results might be related to different templates used to stabilize SeNPs. The cell cycle is primarily driven by cyclin-dependent kinases (CDKs) and cyclins (39). The complexes including cyclin D–CDK4/6 and cyclin E–CDK2 drive the transition from G1 to S phase (40). The cyclin D–CDK4/6 is essential for entering G1 phase, while the cyclin E–CDK2 regulates the induction of DNA synthesis in late G1 and early S phases (37). The INK4 proteins specifically interfere with the association between cyclin D and CDK4/6, whereas the Cip/Kip family of inhibitors such as p21 and p27 inhibit cyclin E–CDK2 activity (41). To explore the molecular mechanism of LNT-SeNPs on cell cycle arrest in HCT-116 cells, the protein expression levels of p21, p27, cyclin D1, CDK2, CDK4, and CDK6 were detected by western blot. As shown in **Figure 7B**, LNT-SeNPs treatment (3, 6, and 12 μM) exhibited a dose-dependent increase in the expression levels of p21 and p27 proteins in HCT-116 cells. Moreover, the down-regulation of cyclin D1, CDK2, CDK4, and CDK6 proteins was observed after LNT-SeNPs treatment. These results confirmed that LNT-SeNPs could arrest HCT-116 cells at G0/G1 phase through modulation of cell cycle regulatory proteins. Several studies have demonstrated that these cell cycle regulatory proteins are closely related to the G0/G1 phase arrest in cancer cells. Xia et al. (42) found that anisomycin-loaded functionalized SeNPs up-regulated the protein levels of p21 and p27 and arrested the cell cycle progression at the G0/G1 phase in HepG2 cells. Lee et al. (43) reported that cannabidiol significantly induced G1 phase arrest in SW620, SW480, and HCT-116 cells by down-regulating the protein levels of cyclin D1, cyclin D3, CDK2, CDK4, and CDK6.

Conclusion

In the present study, SeNPs stabilized by LNT with a mean size of ∼59 nm and could bind with LNT *via* hydrogen bonding interactions. The well-dispersed LNT-SeNPs presented zero-valent, amorphous, and spherical structures and had good stability at 4°C. Compared with SeMet and Na_2SeO_3, LNT-SeNPs exhibited good selectivity between cancer and normal cells. Among five colon cancer cells, LNT-SeNPs showed the highest sensitivity toward HCT-116 cells. Moreover, LNT-SeNPs inhibited the anti-proliferation of HCT-116 cells by regulating mitochondria-mediated apoptotic pathway and inducing cell cycle arrest at G0/G1 phase. The present study provides valuable scientific evidence for the application of LNT-SeNPs in the chemoprevention of CRC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

XG: writing – original draft, methodology, software, data curation, and funding acquisition. YY and XC: investigation. XL: methodology and validation. XY: funding acquisition. C-TH: writing – review and editing. BL and ZC: funding acquisition and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author XG was employed by the company Guangdong Yuewei Edible Fungi Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Feeding foliar nano-selenium biofortified panax notoginseng could reduce the occurrence of glycolipid metabolism disorder in mice caused by high-fat diets

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Nano-selenium (nano-Se) has been extensively explored as a biostimulant for improving the quality of grain crops. However, there are few reports about the effect on the medicinal components of Chinese herbal medicine cultured with nano-Se. Here, we sprayed nano-Se during the cultivation of Panax notoginseng (SePN), and measured the changes of medicinal components compared with conventional Panax notoginseng (PN). Furthermore, we identified a more pronounced effect of SePN on reducing obesity in animals compared with PN. By measuring antioxidant capacity, histopathology, gene expression related to glycolipid metabolism, and gut microbiota composition, we propose a potential mechanism for SePN to improve animal health. Compared with the control groups, foliar spraying of nano-Se increased saponins contents (Rb2, Rb3, Rc, F2, Rb2, and Rf) in the roots of Panax notoginseng, the content of Rb2 increased by 3.9 times particularly. Interestingly, animal studies indicated that taking selenium-rich Panax notoginseng (SePN) can further ameliorate liver antioxidation (SOD, MDA, and GSH) and enzyme activities involved in glycolipid metabolism (ATGL and PFK). It also relieved inflammation and regulated the expression of genes (*MCAD*, *PPAR-α*, and *PCSK9*) related to fatty acid oxidation. The abundance ratio of *Firmicutes/Bacteroides* and beneficial bacteria abundance (*Bifidobacterium*, *Butyricimonas*, and *Parasutterella*) in gut microbiota were improved relative to the control. In summary, the application of nano-Se on PN may effectively raise the content of Panax notoginseng saponins (PNS) and immensely lower the risk of metabolic disorders of glycolipids.

KEYWORDS

nano-selenium, high-fat diet, Panax notoginseng, glycolipid metabolism, gut microbiota

Introduction

At present, more than 600 million individuals are obese, accounting for 39% of the world's adult population (1). Obesity raises the risk of a variety of diseases, including insulin resistance, cardiovascular disease, liver disease, and cancer (2). Obesity is frequently assumed to be caused by a high-fat and high-calorie food supply (3). People progressively recognize the harm of the high-fat diet (HFD) and the importance of a scientific diet, therefore they focus on enhancing bodily function through the use of functional foods (4, 5). Currently, Chinese herbs such as Panax ginseng, Panax notoginseng, and Chinese yam have been experimentally validated for reducing the risk of cardiovascular disease in animal studies (6–8).

Panax notoginseng (PN) is a perennial Chinese herbal medicine with Panax notoginseng saponins (PNS) as its principal functional component. PNS are useful for hemostasis, anti-inflammation, analgesia, and the prevention of cardio-cerebrovascular diseases (9). PNS can dramatically up-regulate the expression of fatty acid β-oxidation related factor mRNA (*MCAD, LCAD*) to intervene with cardiomyocyte hypertrophy by regulating energy metabolism in the field by lowering blood sugar and blood lipids (10). PNS has also been demonstrated to boost the expression of *CPT-1A* in T cells, increasing their fatty acid oxidation metabolism, and promoting the differentiation of mice CD_4^+T cells into Treg cells (11). In a nutshell, PN which can reduce blood sugar and blood lipid levels should be investigated further. However, Chinese herbal medicine is vulnerable to abiotic (heavy metals, drought, flooding, climate change, pesticides) and biotic stress (insects and pathogenic bacteria), which affects the composition and production of medicinal components (12).

Exogenous hormones such as jasmonic acid, salicylic acid, and melatonin have been shown in several studies to effectively alleviate the deleterious effects of biotic and abiotic stress on PN, as well as boost PN biomass and antioxidant capacity (13–15). However, there are few investigations on the intervention of Se with a strong antioxidant capacity of the medicinal components of the PN. Se exerts biological benefits as a component of the antioxidant enzyme glutathione peroxidase (GPX) (16). Foliar Se treatment reduced oxidative stress damage while also increasing grain yield and Se contents (17). Maassoumi et al. (19) found that Se can up-regulate triterpenoid saponins, soluble sugar, amino acids, and exopolysaccharides contents in Astragalus (18). Compared with inorganic and organic Se, nano-Se has high bio-availability, superior stability, minimal toxicity, and great free radical scavenging capabilities. Studies showed that nano-Se may be utilized as biofortifiers and stimulators, and its effect on plant antioxidant metabolism was related to primary and secondary metabolites (20). Our previous studies demonstrated that foliar application of nano-Se regulated hormone pathway, phenylpropane pathway, volatile organic compounds, antioxidants enzymes, and secondary metabolites to strengthen the quality and resistance in various crops (21–23). Medical studies revealed that the protective mechanism of Se on the human body includes inhibition of oxidative stress, endothelial dysfunction, protection of vascular cells from apoptosis, calcification, and regulation of inflammation (24). Long-term Se deficiency in the human body can harm the cardiovascular system and lead to myocardial infarction (25). However, few researchers have studied the mechanism of how nano-Se improves the quality of traditional Chinese medicine PN and the level of glycolipid metabolism after ingesting nano-Se-cultivated PN.

Hence, the saponins contents, physiological and biochemical indexes, antioxidant capacities, enzymes activities, gene expression, pathological analysis, and gut microbiota connected with glycolipid metabolism were targeted determination to explore nano-Se foliar applications in PN acts the effect on glycolipid metabolism in mice.

Materials and methods

Synthesis and characterization of nano-se

The synthesis method refers to the previous research (26). The 1% chitosan solution was prepared for the pre-solution, and then 20 mM of selenium dioxide solution (i.e., selenite solution) was slowly added to 20 mL of the pre-solution. The nanoscale dispersed selenite colloidal solution was obtained by continuous stirring at 500 rpm and 25°C. Slowly add 4 ml 1% ascorbic acid solution and stir continuously at 25°C at the speed of 500 rpm for 3 h until the solution color changes to transparent red, which means that the synthesis of nanometer selenium is over. For characterization data, please refer to our previous article (22).

Cultivation and processing of SePN

The SePN plants were grown in Kunming, Yunnan Province, China. The cultivation method is to select 1-year-old PN seedlings. Nano-Se is sprayed once a month from May to November, and root samples of PN were collected in December. According to the recommended dosage of PN in Chinese Pharmacopeia and the rule of body surface area, the roots PN were ground into powder by a mill and added into HFD to make the mass fraction of PN 6 g/Kg. All reagents needed in the experiment were purchased from commercial channels.

Sample preparation of PN

The 0.1 g mashed PN powder was weighed in a 2 ml centrifuge tube. The saponin was extracted by adding the 1 ml solution (70:30% V/V, methanol/water). The mixture was shaken for the 2 min on the VX-III multi-tube eddy current meter (Beijing Tajin Science and Technology, Beijing, China) and centrifuged for the 5 min at 10,000 rpm. Then, 1 ml supernatant was transferred to a 2 ml centrifuge tube containing 50 mg C18. The tube was vortexed for 2 min before being centrifuged at 10,000 rpm for 5 min. Finally, the supernatant was filtered through a 0.22 μm nylon filter and transferred into an autosampler glass vial for the saponin measurement.

Relative quantification of saponins by HPLC–MS/MS

The HPLC–MS/MS system comprised an Agilent Series 1,290 ultra-performance liquid chromatography system and an Ultivo triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation was performed on a ZORBAX Eclipse Plus C18 chromatography column (2.1 × 50, 1.8 μm, Agilent) using a gradient elution of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The gradient profile was optimized as below, 0–10 min: 5–95% B, 10–12 min: 95–5% B, and 12–15 min: 5% B. The column temperature was maintained at 40 °C, and the injection volume was 2 μL. The composition changes of PNS are shown in Supplementary Table S1. The MRM parameters of each analytes are listed in Supplementary Table S2.

Animals and treatment

Thirty 2-week-old C57 male mice were randomly divided into three groups with 10 mice in each group. They were placed in a stable environment with a temperature of 25 ± 2°C, and humidity of 50 ± 5% with 12 h light/12 h dark cycle. Mice were fed with HFD mixed with PN/SePN and free drinking water for 8 weeks. Body weight and food intake were recorded every 7 days. The mice were fasted for 1 day after collecting their fecal flora, and dissected after fasting to collect liver, heart and serum samples. These samples were collected and stored in −80°C. All experimental operations were approved by the independent Animal Ethical Committee of China Agricultural University.

Biochemical parameters assay of serum

Serum biochemical indexes include aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), glucose levels (GLU), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C). They were measured using their respective assay kits (Nanjing Jiancheng Bioengineering Institute, China).

Biochemical indexes assay of liver

The detection of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) can reflect the oxidative stress indexes of the liver. Phosphofructokinase (PFK), hydroxymethylglutaryl CoA reductase (HMG-CoAR), and adipose triglyceride lipase (ATGL) were detected to reflect the enzyme activity of liver lipid metabolism. These indexes were measured by using the test kit of Nanjing Jiancheng Bioengineering Research Institute (China).

Histopathological analysis of liver and colon

A small piece of liver and colon tissue (about 50 mg) was fixed with a 4% formaldehyde solution. After being embedded in dehydrated paraffin, the tissue was cut into 5 mm slices and then stained with hematoxylin-eosin. Histopathological images were collected by Olympus BX51 imaging system (Olympus Corporation, Japan) and analyzed by Image pro plus 6.0 software (Media Cybernetics, USA).

Total RNA extraction and reverse ranscription

Total RNA was extracted using Trizol-A+ reagent (Tiangen Biotech Co., LTD., Beijing, China). The total RNA was reverse transcribed into cDNA using the FastQuant RT kit (Tiangen Biotech Co., LTD., Beijing, China). All cDNA was stored at −20°C for further testing.

RT-qPCR analysis

Analysis of reverse-transcribed data with Thermofisher 7,500 instrument. The mRNA levels of genes were quantified and normalized against the housekeeping gene *β-actin*, according to the $2^{-\Delta\Delta CT}$ method (27). The sequence information of all primers is listed in Supplementary Table S3.

Extraction and analysis of microbial DNA

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross,

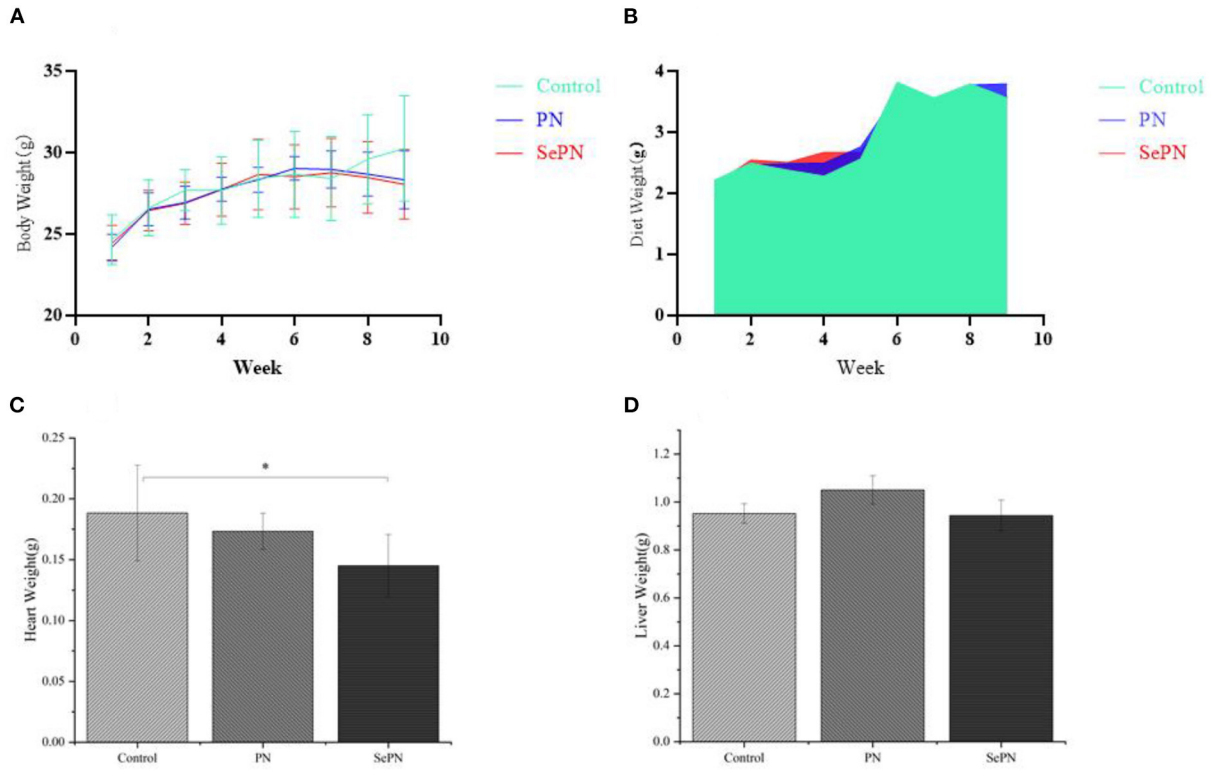

FIGURE 1
Body weight, diet and organ weight of mice. **(A)**. Body weight after 9 weeks of feeding high-fat diet (Control), Panax notoginseng (PN), and selenium-rich Panax notoginseng (SePN). **(B)**. Dietary intake within 9 weeks. **(C,D)**. Liver and heart weight after 9 weeks of feeding PN/SePN. Data are expressed as the mean ± SD. *$P < 0.05$ compared with the control group ($n = 9$).

GA, USA), and stored at −20°C before further analysis. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Amplification system including 5×reaction buffer 5 μL, 5×GC buffer 5 μL, dNTP (2.5 mM) 2 μL, Forward primer (10 uM) 1 μL, Reverse primer (10 uM) 1 μL, DNA Template 2 μL, ddH_2O 8.75 μL, Q5 DNA Polymerase 0.25 μL. Amplification parameters include initial denaturation 98°C 2 min, denaturation 98°C 15 s, annealing 55°C 30 s, extension 72°C 30 s, final extension 72°C 5 min, 10°C hold. 25-30 cycles dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). PCR amplicons were purified and quantified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA), respectively. Amplicons were pooled in equal amounts, and pair-end 2×250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Statistical analyses

All results are represented as the mean ± SD. The graphical illustrations were processed by Origin64 and GraphPad Prism 9 (OriginLab (MA), GraphPad (CA), USA), and the statistical analyses were performed using SPSS v19.0 (IBM, USA). One-way ANOVA and Tukey were used to test the significant differences of variables among different groups. Significant differences in gut microbiota between treatments were analyzed using principal coordinates analysis (PCoA). QIIME2 (2019.4) software was used to analyze the taxonomic composition. Community composition difference was analyzed by the LEfSe test.

Results

Body weight, diet and organ weight of mice

Compared with the control group, there was a trend of weight loss during the 7th and 9th weeks and no alteration in

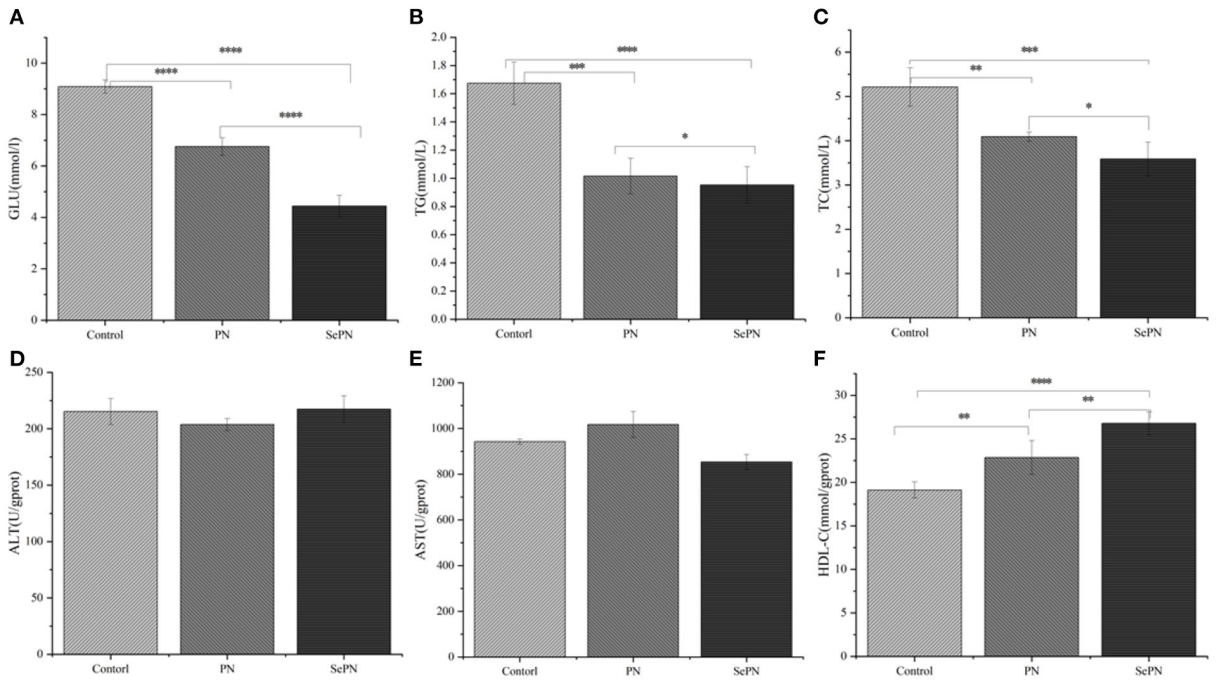

FIGURE 2
Glucolipid metabolism physiological indexes in serum of mice. **(A–C)**. The content of glucose (GLU), triglyceride (TG), and total cholesterol (TC) in the serum. **(D,E)**. The level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). **(F)**. High-density lipoprotein cholesterol (HDL-C). Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ compared with the control group ($n = 5$).

daily food intake. There was no significant difference between the groups (Figures 1A,B). In addition, the heart weight of the mice in the SePN group decreased significantly (Figure 1C), but the liver weight did not change (Figure 1D).

Glucolipid metabolism physiological indexes in serum of mice

We detected the physiological and biochemical indexes related to glycolipid metabolism and found that ingesting SePN could significantly reduce the GLU content of mice compared with PN (Figure 2A). The same results were also reflected in physiological and biochemical indicators related to lipid metabolisms, such as TG and TC (Figures 2B,C). Furthermore, the treatment groups (PN, SePN) did not reduce the level of ALT and AST levels (Figures 2D,E). Taking SePN increased HDL-C contents in serum to a higher extent (Figure 2F).

Antioxidant and enzymes activities of glucolipid metabolism in the liver

After testing the antioxidant stress index of mouse liver, we found that taking PN and SePN could alleviate the oxidative damage caused by reactive oxygen species and reduce MDA levels to some extent (Figure 3B). The treatment groups significantly increased the activity of GSH, but there was no difference between the treatment groups (Figure 3C). What's more, we detected some rate-limiting enzymes of glycolipid metabolism, and we found that compared with the PN group, ingesting SePN could reduce the activity of cholesterol synthase to a certain extent (Figure 3D), increase the activity of ATGL and PFK in glucolipid metabolism (Figures 3E,F). These results showed that compared with PN, SePN could relieve liver injury by improving the ability of antioxidant stress, and protecting the function of glucolipid metabolism of the liver.

Representative images of H&E staining in colon and liver sections

Compared with the H&E staining images of the control group (Figure 4), SePN treatment groups could further increase the number of goblet cells and relieve HFD-induced colon inflammation. Meanwhile, the H&E staining image of the liver indicated that the lymphocytes were infiltrated, activated, and cavitated in the control groups, but the treatment groups could significantly reduce the chronic inflammation hazardous effect to the liver.

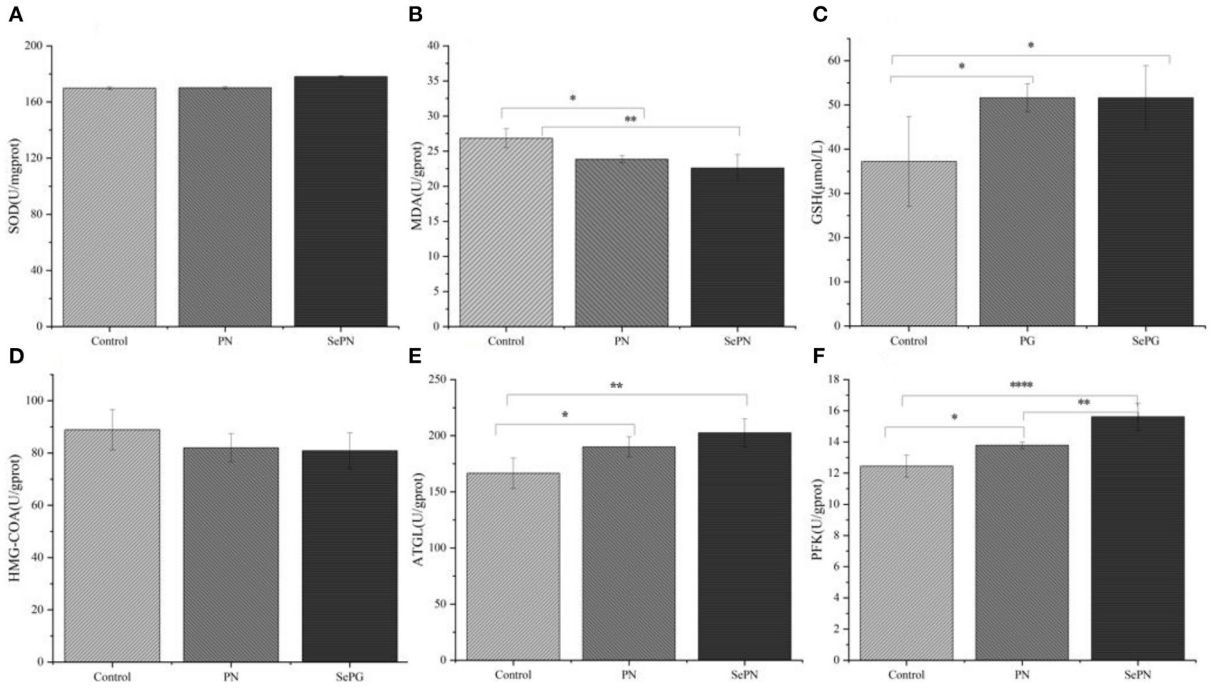

FIGURE 3
Antioxidant and enzyme activities of glucolipid metabolism in the liver. **(A)** Liver superoxide dismutase (SOD) levels. **(B)** Liver malondialdehyde (MDA) levels. **(C)** Liver glutathione (GSH) levels. **(D–F)** Effects of PN/SePN on liver hydroxymethylglutaryl CoA reductase (HMG-CoAR), adipose triglyceride lipase (ATGL), and phosphofructokinase (PFK). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ compared with the control group ($n = 5$).

Detection of the expression of lipid metabolism related genes by RT-PCR

CPT1 is the key enzyme for the entrance of lipid into mitochondria, and oxidative enzymes MCAD, and LCAD will determine the lipid used for the TCA cycle in oxidation. We found that SePN could increase the expression of *CPT1* gene by nearly two times (Figure 5A). Secondly, we detected the expression of *LCAD* and *MCAD*, the other two key genes of fatty acid β-oxidation, and found that the expression of *MCAD* was enhanced, which meant that the ability of medium-chain fatty acid β-oxidation metabolism was enhanced. However, it had no significant effect on the expression level of *LCAD*, which meant that SePN could specifically increase the activity of fatty acid oxidase (Figures 5B,C). *PPAR-α* and *PPAR-γ* play key roles in maintaining glucose and lipid homeostasis by modulating gene expression. Our studies found that SePN significantly up-regulated the expression of *PPAR-α*, but it had no significant effect on *PPAR-γ* expression (Figures 5D,E). In addition, SePN significantly down-regulated the expression of *PCSK9* (Figure 5F).

Changes of gut microbiota abundance in mice

Sequencing analysis of 16S rRNA gene reveals effects on the composition of gut microbiota. PCoA plots indicated a significant difference in the composition of the gut microbiome between the control and treatment groups (Figure 6A). What's more, population abundance analysis was carried out at the phylum level. The results showed that the gut microbiome was mainly composed of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Figure 6B). The treatment groups increased the relative abundance of *Bacteroidetes*, which decreased the *Actinobacteria* abundance and *Firmicutes*/*Bacteroides* abundance ratio. To clarify the changes in microbes' abundance among different treatment groups, we performed the LEfSe to find microbes with significant differences (Figure 6C). Notably, in the SePN group, the most significant increase in the abundance of *Bacteroidetes*, *Butyrimionas*, *Bifidobacterium*, and *Parasutterella*, and reduce the abundance of *Tenericutes* (Supplementary Figure S1).

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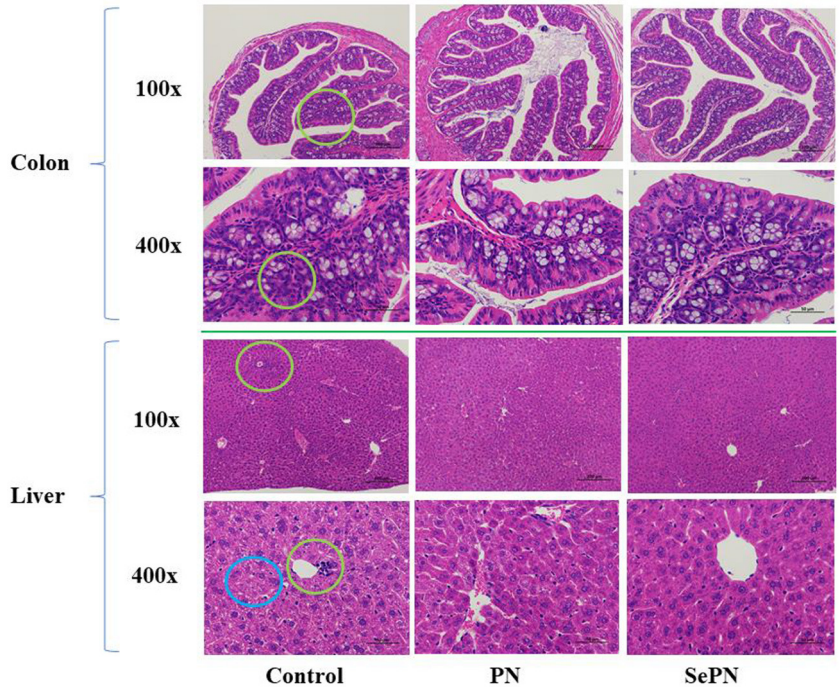

FIGURE 4
Representative images of H&E staining in colon and liver sections. We use green circles to indicate inflammatory cell infiltration and blue circles to indicate cell vacuolation.

Discussion

Nano-selenium biofortification acts the accumulation of Panax notoginseng saponins (Rb3, Rc, F2, Rb2, and Rf). According to various research, these saponins have the potential to alleviate cardiovascular and cerebrovascular disorders induced by HFD (28–30). Studies showed that selenium deficiency induces Keshan disease, which is characterized by cardiac hypertrophy and increased heart weight (31). We found that the heart weight of mice was reduced after ingesting SePN, which is most likely due to the synergistic effect of Se with saponins. The risk of cardiovascular and cerebrovascular diseases is directly connected to blood sugar and blood lipid contents. The TG, TC, HDL-C, GLU, ALT, and AST levels in the mice serum can directly reflect whether Panax notoginseng protects liver glycolipid metabolism. Our studies discovered that in the treatment groups, TG, TC, and GLU contents declined while HDL-C rose, implying that blood viscosity decreased, but the blood flow velocity improved, lowering the risk of hyperglycemia and hyperlipidemia. However, the activities of ALT and AST in serum did not change considerably, indicating that taking SePN at the prescribed dose every day could not successfully treat the liver impairment produced by long-term HFD.

To further explore the effect of taking SePN on the liver, we detected the related indexes of oxidative stress and glycolipid metabolism. The results showed that the treatment groups might increase the activity of GSH and decrease MDA levels induced by lipid peroxidation, indicating that SePN could protect liver function by improving antioxidation ability. *HMG-CoAR* is involved in cholesterol synthesis and LDL catabolism in serum. The level of *HMG-CoAR* in the treatment groups decreased relatively, but the difference was not statistically significant. It could be inferred that PNS increased the HMG-CoAR activities, inhibited the cholesterol synthesis metabolism of the liver, and promoted the catabolism of LDL, which were beneficial to reduce cholesterol levels in serum. Secondly, the increasing types of saponins (Rb2, etc.) in SePN might not further inhibit cholesterol synthesis metabolism. ATGL and PFK were used as rate-limiting enzymes to chew through TG and GLU, respectively. Compared with the control group, the enzyme activities of ATGL and PFK decreased significantly in the treatment groups. SePN group more efficiently promoted aerobic respiration of GLU and the mobilization of fat. Furthermore, there was a significant positive correlation between PNS contents and ATGL and PFK enzymes, which meant PNS could activate the synthesis of ATGL and PFK.

The study examined the expression level of lipid metabolism-related factors in the liver to further explore

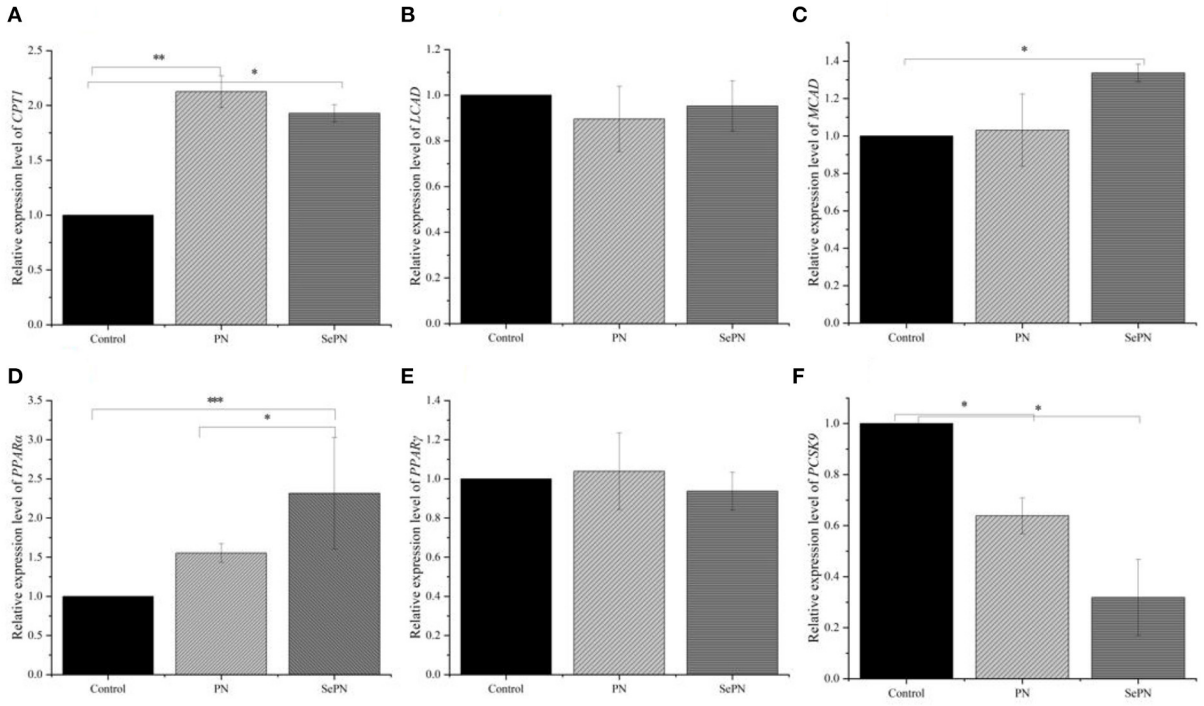

FIGURE 5
Detection of the expression of glucolipid metabolism related genes by RT-PCR. **(A)**. The relative abundance of mRNA of *CPT1*. **(B,C)** The relative abundance of mRNA of *LCAD* and *MCAD*. **(D,E)** mRNA relative abundance of *PPAR-α and PPAR-γ*. **(F)** The relative abundance of mRNA of *PCSK9*. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and compared with the control groups ($n = 3$).

the changes in fatty acid metabolism function. *CPT1* is a key factor that promotes the transfer of fatty acids from cell fluid to mitochondrial inner membrane for β-oxidation (32). Inhibition of *CPT1* expression can lead to lipid accumulation and insulin resistance (33, 34). In the liver, peroxisome proliferators activate receptors α and γ (*PPAR-α, PPAR-γ*) to regulate the homeostasis of lipid metabolism (35). Among them, *PPAR-α* is involved in regulating the enzyme activity of gluconeogenesis, lipoprotein synthesis and transport determines the capacity of hepatic fatty acid oxidation in the liver (36). *PPAR-γ* plays an important role in adipogenesis, lipid metabolism, insulin sensitivity, and immune regulation (35). The activation of *PPAR-γ* is adipogenic, and the increased expression of *PPAR-γ* in the liver will lead to steatosis (37). MCAD and LCAD catalyze the first step of fatty acid oxidation and determine the lipid used for the TCA cycle (38, 39). Low-density lipoprotein receptor (LDLR) can bind to LDL and reduce LDL contents in serum (40). Subtilisin 9 (*PCSK9*) can negatively regulate the expression of *LDLR*, resulting in hypercholesterolemia (41). Our studies indicated that PNS could affect *PPAR-α* and *PCSK9*-related pathways to reduce fatty acid and LDL levels, and SePN would produce better results. Furthermore, studies indicated that SePN with higher PNS contents could reduce the accumulation of lipids and maintain normal glucolipid metabolism by promoting the utilization of fatty acids, thus reducing the contents of blood sugar and blood lipids.

HFD can significantly decrease the number of goblet cells in the colon, which can secrete mucin to form a mucosal barrier, protect epithelial cells, and reduce the risk of colonic inflammation (42). Compared with PN, SePN could significantly increase the number of goblet cells in the colon to resist endogenous or exogenous stimulation and reduce lymphocyte infiltration and vacuolization in the liver. Considering the increase in antioxidant level, we believed that SePN could protect the liver and reduce the chronic inflammation and metabolic disorder caused by lipid peroxidation, and the mechanisms might be due to the combined effect of high accumulation of PNS and Se.

Furthermore, we further investigated the effects of SePN on gut microbiota. HFD decreases *Bacteroides* levels while increasing the amount of *Firmicutes*, which was a characteristic of the gut microbiota of obese people, according to 16s rRNA sequencing (43). The abundance of *Bacteroides* rose dramatically after the treatment of PN and SePN therapy, but the abundance of *Actinobacteria* declined comparatively. A lower *Firmicutes* / *Bacteroides* ratio indicates a lower risk of obesity (44). Moreover, we used the LEfSe further analyze the differences in gut microbiota composition among groups. Studies showed

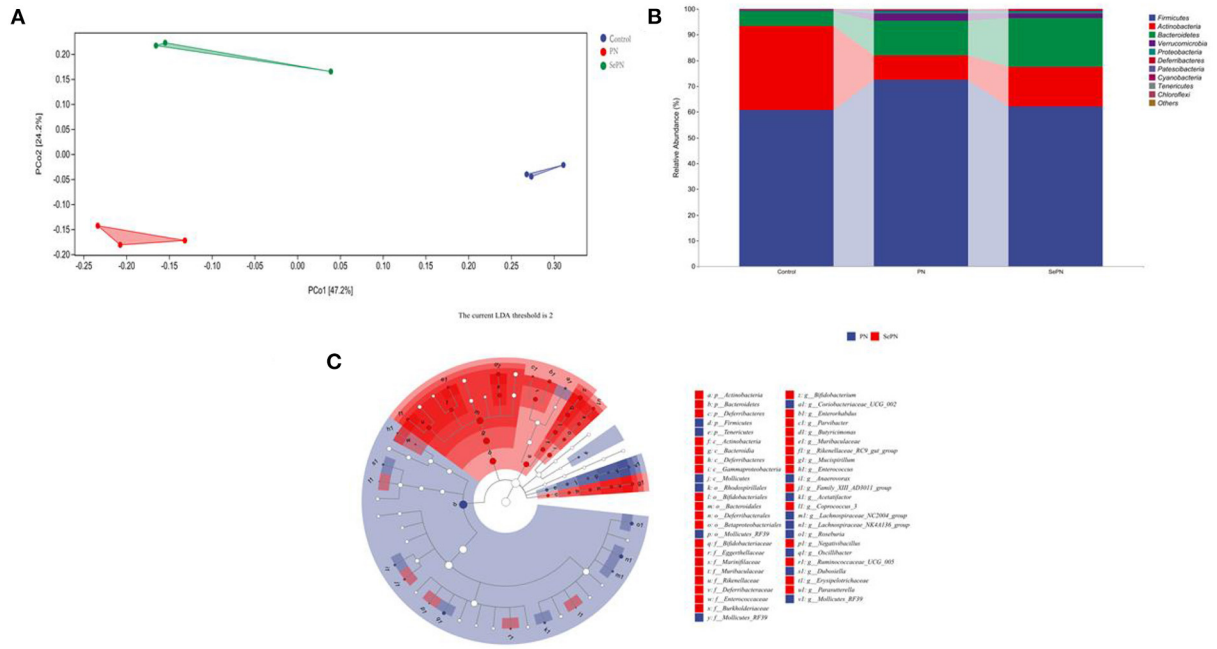

FIGURE 6
Changes of gut microbiota abundance in mice. **(A)** The principle coordinate analysis (PCoA) plot for the samples in the different groups. **(B)** The gut microbiota composition at the phylum level. **(C)** Taxa with significant differences between groups based on linear discriminant analysis (LDA) effect size.

that *Butyricimonas*, which can produce butyric acid to improve the inflammatory response (43, 45, 46), *Bifidobacterium*, which produces acetic acid to regulate the induction of cholesterol biosynthesis (43, 47), and *Parasutterella*, which is involved in bile acid homeostasis maintenance and cholesterol metabolism (43, 48), increased their abundance after taking SePN. Compared with PN group, taking SePN increased the relative abundance of *Muribaculaceae*, *Rikenellaceae*, *Erysipelotrichaceae* (Supplementary Figure S1). At present, studies showed that the increase in the abundance of these microorganisms is negatively correlated with the risk of disease induced by HFD (49–52). Interestingly, some studies showed that dietary selenium supplementation can improve their relative abundance (53, 54). Therefore, the increase of PNS and selenium content jointly regulate the gut microbiota and reduce the negative effects of HFD. These results confirmed that SePN had a better role in regulating glycolipid metabolism and relieving inflammatory reactions. There are significant changes in glucolipid metabolism in mice juvenile to young adulthood. These changes and the potential effects of selenium absorption and transmission regulation of glucolipid metabolism in mice need to be further studied.

Conclusion

In conclusion, we combined plant and animal experiments together and hoped to fully demonstrate the meaning of the nano-selenium biofortification on Chinese herbal medicine Panax notoginseng by the target determination and 16S rRNA gene sequence analysis. Our study indicated that taking SePN instead of PN might boost antioxidant capacity, glucolipid metabolism enzyme activities, *PPARα* and *PCSK9* pathway regulation, and gut microbiota improvement linked to glucolipid metabolism, lowering the risk of hyperglycemia, hyperlipidemia, and inflammatory reactions induced by the HFD.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: 185824296@qq.com, https://datadryad.org/stash/share/wNEl5d0IV0JZZrwQgHbSDHyBiFaCSbAu2D71_cNlmMU.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care Program, China Agricultural University.

Author contributions

QD planned the study and prepared the manuscript and analyzed the data. SY, WZ, and DL designed the research. QD, YW, ST, and PM carried out experiments. DL, SY, and CZ contributed to the preparation of the manuscript. WZ provided platform and technical support. SZ provided the planting technology of Panax notoginseng and completed the sample collection. CP modified the manuscript and approved the implementation of this experiment. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.973027/full#supplementary-material

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A new selenium source from Se-enriched *Cardamine violifolia* improves growth performance, anti-oxidative capacity and meat quality in broilers

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Background: *Cardamine violifolia* (Cv) is a kind of selenium-enriched plant which contains high levels of organic selenium (Se) such as selenocysteine and methylselenocysteine. This study was conducted to investigate the effects of this new source of Se on the growth performance, anti-oxidative capacity and meat quality in broilers compared with other frequently-used Se sources.

Methods: A total of 240 broilers were allotted into 4 treatments: (1) Control group (Se free diets, CON); (2) Sodium selenite group (0.3 mg/kg Se sourced from Na_2SeO_3 diets, SeNa); (3) Selenium yeast group (0.3 mg/kg Se sourced from Se-Yeast diets, SeY); (4) Plant Se group (0.3 mg/kg Se sourced from Cv diets, SeCv). The whole study lasted 42 days and was divided into 2 stages (1–21 d as earlier stage and 22–42 d as later stage).

Results: The results showed that the broilers fed SeCv diets had improved average daily gain and the ratio of feed to gain compared to the broilers fed SeNa and SeY diets during the earlier stage. However, there was no significant difference in growth performance of broilers fed these 3 sources of Se diets during the whole period. The broilers fed SeCv diets had improved intestinal mucosal morphology on d 21 and 42. Enhanced liver total anti-oxidative capacity was observed from the broilers fed SeCv diets compared with the other 2 Se sources diets on d 21. Furthermore, lower liver malondialdehyde contents were determined from the broilers fed SeCv and SeY diets compared with SeNa diets. At last, the broilers fed SeCv had increased redness in thigh muscle and decreased cooking loss in both breast and thigh muscle compared with the boilers fed SeNa diets. However, the broilers had similar meat quality between SeCv group and SeY group.

Conclusion: In conclusion, these results demonstrated that SeCv was a well-organic Se source for broilers.

KEYWORDS

***Cardamine violifolia,* broilers, selenium, antioxidant, performance, meat quality**

Introduction

Selenium (Se) is an essential trace element for humans and animals as its crucial functions in antioxidant defense, immune enhancement and so on (1, 2). Se plays its biological function as the core constituent of selenocysteine ($SeCys_2$) and selenomethionine (SeMet) which are constituent of antioxidant enzymes such as glutathione peroxidase (GSH-PX) (3, 4). The frequently-used sources of Se for feed additives in poultry diets include sodium selenite (Na_2SeO_3) and selenium yeast (SeY). Similar to other trace elements, the bioavailability of Se closely depended on the chemical forms (5). It is generally believed that organic Se sources have lower toxicity, higher bioavailability and better antioxidant properties compared to its inorganic sources (6, 7).

Cardamine violifolia (Cv) is a recently discovered Se hyperaccumulating Brassicaceae plant found in Enshi, Hubei, China (8). Cv can efficiently transform inorganic Se into organic Se through its particular metabolic pathways (9). Meanwhile, Cv has a super high Se tolerance and it can accumulate Se above 1,400 mg Se per kg dry weight (10, 11). The main forms of Se existed in Cv are Se-enriched proteins such as $SeCys_2$, methylselenocys-teine (MeSeCys) and SeMet (12). The edibility and Se accumulation ability of Cv make it as a potential source of Se supplementation for feed additives. However, there are few studies focused on the effects of Se-enriched Cv (SeCv) on the growth performance and health status in livestock and poultry.

Therefore, this study was aimed to explore the effects of this new Se source—SeCv on the growth performance, anti-oxidative capacity and meat quality in broilers compared with other frequently-used Se sources—SeNa and SeY, respectively.

Materials and methods

Animal and experimental design

Animal trial was conducted according to the Animal Scientific Procedures Act 1986 (Home Office Code of Practice. HMSO: London January 1997) and EU regulation (Directive 2010/63/EU). This experimental protocol (No. WPU202111049) used in this study was approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Wuhan, China). A total of 240 one-day-old, male (initial body weight 45.34 ± 0.67 g) ROSS 308 broiler chicks were obtained from the Xiangyang Charoen Pokphand Co., Ltd., Xiangyang, Hubei, China. All birds were randomly assigned to 1 of 4 dietary treatments with 6 pens per treatment and 10 chicks per pen. The treatments were designed as (1) Control group (Se free diets, CON); (2) Sodium selenite group (0.3 mg/kg Se sourced from Na_2SeO_3 diets, SeNa); (3) Selenium yeast group (0.3 mg/kg Se sourced from Se-Yeast diets, SeY); (4) Plant Se group (0.3 mg/kg Se sourced from Cv diets, SeCv). All diets were formulated to meet the nutritional levels for broiler chickens recommended by the National Research Council (1994) (13). The chickens were fed earlier diets from d 1 to 21 and later diets from d 22 to 42. The composition and nutritional level of the experimental diets is shown in Table 1. All chicks were allotted in wire-floored cages (120 × 120 × 60 m^3) in an environmentally controlled room with continuous light and had *ad libitum* access to feed and water. The ambient temperature was maintained at 36°C at the start of experiment and was decreased as the birds progressed in age. The relative humidity was set at 45–55% and was kept within this range.

TABLE 1 Composition and nutrient contents of basal diets for the broilers (as fed basis).

Ingredients, %	D 1–21 earlier stage	D 22–42 later stage
Corn	55.59	60.80
Soybean meal	36.85	32.50
Soy oil	3.77	3.45
Calcium hydrophosphate	1.65	1.15
Limestone	1.37	1.43
Salt	0.30	0.30
DL-methionine	0.17	0.07
Vitamin and mineral premix[a]	0.30	0.30
Nutrient contents, %[b]		
Metabolizable energy, kcal/kg	3,000	3,050
Crude protein	21.10	19.54
Ca	1.02	0.92
Non-phytate phosphorus	0.45	0.35
Lysine	1.14	1.03
Methionine	0.50	0.38
Threonine	0.80	0.74

[a]Premix Supplied per kg Diet: Vitamin A, 11,000 IU; Vitamin D, 3,025 IU; Vitamin E, 22 mg; Vitamin K_3, 2.2 mg; Thiamine, 1.65 mg; Riboflavin, 6.6 mg; Pyridoxine, 3.3 mg; Cobalamin, 17.6 μg; Nicotinic Acid, 22 mg; Pantothenic Acid, 13.2 mg; Folic Acid, 0.33 mg; Biotin, 88 μg; Choline Chloride, 500 mg; Iron, 48 mg; Zinc, 96.6 mg; Manganese, 101.76 mg; Copper, 10 mg; Iodine, 0.96 mg; Cobalt, 0.3 mg. [b]The Nutrients Contents Were Analyzed Values Except Metabolizable Energy and Non-phytate Phosphorus Which Were Calculated Values.

Se-enriched Cv used in this study was provided by Enshi Se-Run Health Tech Development Co., Ltd, Enshi, Hubei, China. This Se-enriched Cv included leaves and stalk with 1,430 mg Se per kg dry weight mainly existed as the forms of $SeCys_2$ and MeSeCys determined by the method of high performance liquid chromatography combined with atomic fluorescence (Figure 1). The sodium selenite and selenium yeast samples were

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; Cd, crypt depth; Cv, *Cardamine violifolia*; F/G, feed-to-gain ratio; GSH-PX, glutathione peroxidases; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutases; T-AOC, total anti-oxidative capacity; Vh, villus height.

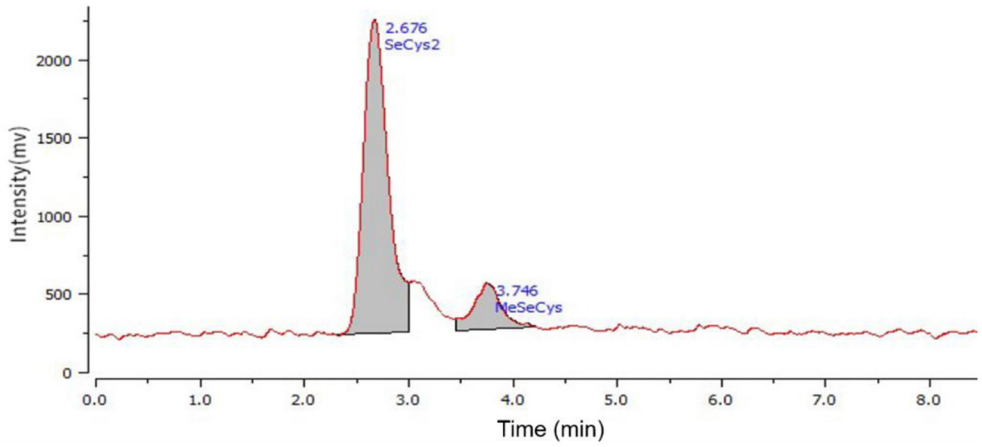

FIGURE 1
Characterization of selenium existed in Se-enriched *Cardamine violifolia*.

commercial products purchased from Angel Yeast Co., Ltd, Yichang, Hubei, China.

Sample collection

All chicks were weighed individually after their arrival from the hatchery. These birds were also weighed on d 21 and 42. Feed bags were weighed at the same time and these data were used to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed-to-gain ratio (F/G). One bird per cage was randomly selected according to the average weight of the broilers in the cage on d 21 and 42, respectively. The intestine, liver and muscle samples were collected. A 2-cm tissue samples of the duodenum, jejunum and ileum were obtained. The intestinal samples were flushed with 0.9% salt solution, fixed with 10% formaldehyde-phosphate buffer and kept at 4°C for microscopic assessment of intestinal morphology. The liver samples were collected and frozen in liquid nitrogen and transferred to a −80°C freezer for anti-oxidative capacity analysis. The broilers euthanized on d 42 were also used to collect breast and thigh muscle samples. The muscle samples were removed on the left side of the broilers for meat quality determination.

Intestinal morphology

After a 24 h fixation, the intestinal segments were dehydrated, embedded, and stained with hematoxylin and eosin. Villus height (Vh) and crypt depth (Cd) were measured at 100 × magnification with a microscope (Olympus CX31, Tokyo, Japan) according to our previous study (14). Ten well-oriented and intact villi were selected and determined using a light microscope with a computer-assisted morphometric system (BioScan Opti-metric; BioScan Inc., Edmond, WA, USA). Vh was measured from the tip of the villus to the villus-crypt junction; Cd was defined as the depth of the invagination between adjacent villi.

Liver anti-oxidative capacity

Total anti-oxidative capacity (T-AOC), activities of glutathione peroxidases (GSH-PX), superoxide dismutases (SOD) and concentrations of malondialdehyde (MDA) in liver were determined by spectrophotometric methods following the instructions of the commercial kits' manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (15).

Meat quality of the breast and thigh muscles

Meat color, including lightness (L*), redness (a*), and yellowness (b*) values, were measured from 3 locations (middle, medial, and lateral) using a Chromameter (CR-410, Konica Minota, Tokyo, Japan) (16). Drip loss for 24 was measured using the plastic bag method as described previously (17). Briefly, a weight of 30 g of breast or thigh muscle sample was weighed and then put in a sealed plastic bag and kept at 4°C for 24 h. After which the samples were taken out of the bags and dried. The drip loss was the difference of the sample weight to the initial sample weight. Cooking loss was measured by a previous study (18). Briefly, a weight of 30 g sample was weighed and cooked at 80°C for 20 min using a water bath. After cooking, the samples were cooled at room temperature and then weighed. Cooking loss was the difference of the sample weight to the initial weight. Shear force were measured by the methods reported previously (19). Briefly, the cooked samples were taken parallel to muscle

TABLE 2 The growth performance of the broilers fed diets containing different sources of selenium.

Items	CON	SeNa	SeY	SeCv	SEM	*P*-value
D 1 Body weight (g)	45.61	45.12	45.50	45.12	0.67	0.925
D 21 Body weight (g)	645^{b}	651^{b}	656^{b}	726^{a}	16	0.027
D 42 Body weight (g)	1982^{b}	2037^{ab}	2021^{ab}	2095^{a}	29	0.043
D 1–21						
Average daily gain (g/d)	28.54^{b}	28.87^{b}	29.07^{b}	32.40^{a}	0.83	0.025
Average daily feed intake (g/d)	44.20	42.72	42.33	42.53	1.13	0.735
Feed to gain ratio	1.55^{a}	1.48^{a}	1.46^{a}	1.32^{b}	0.05	0.012
D 22–42						
Average daily gain (g/d)	63.66	65.98	64.99	65.21	1.22	0.187
Average daily feed intake (g/d)	117.4	114.1	114.4	114.8	1.16	0.325
Feed to gain ratio	1.85^{a}	1.73^{b}	1.76^{ab}	1.76^{ab}	0.03	0.038
D 1–42						
Average daily gain (g/d)	46.10^{b}	47.43^{ab}	47.03^{ab}	48.81^{a}	0.68	0.046
Average daily feed intake (g/d)	80.81	78.39	78.34	78.67	0.99	0.568
Feed to gain ratio	1.75^{a}	1.65^{b}	1.67^{b}	1.61^{b}	0.02	0.002

$N = 6$ (10 birds per cage). The same letter on the shoulder of the mean in the same line indicates that the difference is insignificant, and the absence of the same letter means that the difference is significant. SEM, standard error of mean.

fibers to measure maximal shear force (TA500 Lloyd Texture Analyzer fitted with a triangular Warner-Bratzler shear, Lloyd instruments, Bognor Regis, UK).

Statistical analyses

All data were analyzed as a randomized block design using the general linear model procedures (GLM) of SAS (SAS Inst. Inc., Cary, NC). Statistical significance was declared at $P < 0.05$. If significant effects were found, individual means were compared using Duncan's multiple comparison tests. Data were presented as means and SEMs.

Results

Growth performance

As shown in Table 2, the body weight on d 21 of the broilers fed SeCv diets was significantly increased compared with the other 3 groups ($P < 0.05$). During the earlier period, the broilers fed SeCv diets had significantly increased ADG and decreased F/G compared with the other 3 groups ($P < 0.05$). During the later period, the broilers fed the 3 sources of Se diets had no significant difference in growth performance ($P > 0.05$). For the whole feeding period, the broilers fed SeCv diets had significantly increased ADG and decreased F/G compared with the broilers fed CON diets ($P < 0.05$). Similarly, the broilers in SeCv group had a significantly increased body weight on d 42 compared with the broilers in CON group ($P < 0.05$).

Intestinal morphology

Table 3 showed the intestinal morphology of the broilers fed diets containing different sources of Se. On d 21, Vh and Vh/Cd of duodenum and ileum in the broilers fed SeCv diets were significantly increased compared with the broilers fed the other 3 diets ($P < 0.05$). Vh and Vh/Cd of jejunum in the broilers fed SeCv diets were significantly increased compared with the broilers fed CON and SeY diets ($P < 0.05$). On d 42, the broilers fed SeCv diets had significantly increased Vh and Vh/Cd of duodenum compared with the other 3 groups ($P < 0.05$). Jejunal Vh and Vh/Cd in the broilers fed SeCv diets were significantly increased compared with the broilers fed CON and SeNa diets ($P < 0.05$). Ileal Vh in the broilers fed SeCv diets were significantly increased compared with the other 3 groups ($P < 0.05$). And ileal Vh/Cd in the broilers fed SeCv diets were significantly increased compared with the CON group ($P < 0.05$). Similar to the above results, the histological appearance showed that dietary SeCv alleviated intestinal mucosal injury of the broilers compared with the other 3 groups on d 21 and 42 (Figures 2, 3).

Liver anti-oxidative capacity

On d 21, the liver T-AOC of broilers fed SeCv diets was significantly increased compared with the other 3 groups ($P < 0.05$, Table 4). The activities of GSH-PX and SOD had no significant difference among the broilers fed different sources of Se ($P > 0.05$). However, the broilers fed the 3 sources of Se diets all had significantly increased activities of GSH-PX and

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TABLE 3 The intestinal morphology of the broilers fed diets containing different sources of selenium.

Items	CON	SeNa	SeY	SeCv	SEM	*P*-value
D 21						
Duodenum						
Villus height (μm)	889^{c}	1003^{b}	1035^{b}	1173^{a}	26	<0.001
Crypt depth (μm)	223	244	255	263	13	0.158
Villus height/crypt depth	3.99^{b}	4.11^{b}	4.06^{b}	4.46^{a}	0.13	0.018
Jejunum						
Villus height (μm)	622^{c}	777^{ab}	757^{b}	827^{a}	24	0.008
Crypt depth (μm)	168	175	181	185	12	0.420
Villus height/crypt depth	3.70^{c}	4.44^{ab}	4.18^{b}	4.47^{a}	0.14	0.042
Ileum						
Villus height (μm)	615^{c}	617^{c}	693^{b}	767^{a}	28	<0.001
Crypt depth (μm)	180	174	190	194	12	0.625
Villus height/crypt depth	3.42^{b}	3.55^{b}	3.65^{b}	3.95^{a}	0.12	0.003
D 42						
Duodenum						
Villus height (μm)	817^{c}	935^{b}	1002^{b}	1143^{a}	35	<0.001
Crypt depth (μm)	274	274	248	251	16	0.418
Villus height/crypt depth	2.98^{d}	3.41^{c}	4.04^{b}	4.55^{a}	0.16	<0.001
Jejunum						
Villus height (μm)	756^{c}	881^{b}	963^{a}	989^{a}	31	0.020
Crypt depth (μm)	252	263	268	267	14	0.622
Villus height/crypt depth	3.00^{c}	3.35^{b}	3.59^{ab}	3.70^{a}	0.15	0.017
Ileum						
Villus height (μm)	625^{c}	684^{bc}	701^{b}	773^{a}	28	<0.001
Crypt depth (μm)	194	203	212	219	14	0.254
Villus height/crypt depth	3.22^{b}	3.37^{ab}	3.31^{ab}	3.53^{a}	0.14	0.038

$N = 6$ (1 bird per cage). The same letter on the shoulder of the mean in the same line indicates that the difference is insignificant, and the absence of the same letter means that the difference is significant. SEM, standard error of mean.

SOD compared with the broilers fed CON diets ($P < 0.05$). Similarly, the broilers fed the 3 different sources of Se all had significantly reduced MDA content ($P < 0.05$). On d 42, the liver T-AOC of broilers fed SeCv diets was significantly increased compared with CON and SeNa group ($P < 0.05$). The activities of GSH-PX and SOD results were similar to the results on d 21. The broilers fed the 3 sources of Se diets all had significantly increased activities of GSH-PX and SOD compared with the broilers fed CON diets ($P < 0.05$). Liver MDA content in the broilers fed SeCv diets were significantly decreased compared with the broilers fed CON and SeNa diets ($P < 0.05$).

Meat quality of breast and thigh muscles

The redness of breast muscle in the broilers fed SeCv diets was significantly higher compared with the broilers fed CON diets ($P < 0.05$, Table 5). The broilers fed the 3 sources of Se diets had no significantly difference in redness, drip loss and shear force in breast muscle ($P < 0.05$). Compared with the broilers fed CON diets, the broilers fed SeCv diets had decreased drip loss, cooking loss as well as shear force ($P < 0.05$). As for thigh muscle, dietary SeCv significantly improved redness compared with the other 3 groups ($P < 0.05$). The broilers fed SeCv diets had significantly reduced drip loss in thigh muscle compared with broiler fed CON diets ($P < 0.05$). Moreover, the broilers fed SeCv diets had significantly reduced shear force in thigh muscle compared with broiler fed CON and SeNa diets ($P < 0.05$).

Discussion

The hypothesis that the new source of Se from SeCv has equal or exceeded effects compared with Se sources from SeNa and SeY on growth performance, anti-oxidative capacity and meat quality of broilers was partly supported by the results that the broilers fed SeCv diets had increased liver T-AOC and reduced MDA content, improved intestinal morphology and reduced cooking loss of breast and thigh muscle than

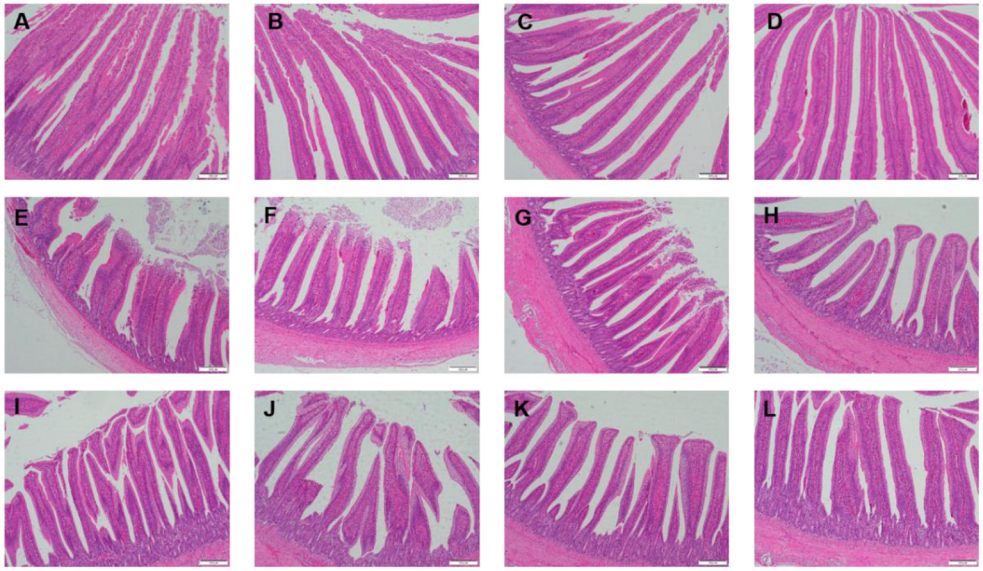

FIGURE 2
Intestinal mucosal histological appearance (hematoxylin and eosin) of the broilers fed diets containing different sources of selenium on D 21. Original magnification 100 ×. Scale bars = 200 μm. **(A–D)** Duodenum histological appearance of the broilers fed CON, SeNa, SeY, and SeCv diets, respectively. **(E–H)** Jejunum histological appearance of the broilers fed CON, SeNa, SeY, and SeCv diets, respectively. **(I–L)** Ileum histological appearance of the broilers fed CON, SeNa, SeY, and SeCv diets, respectively.

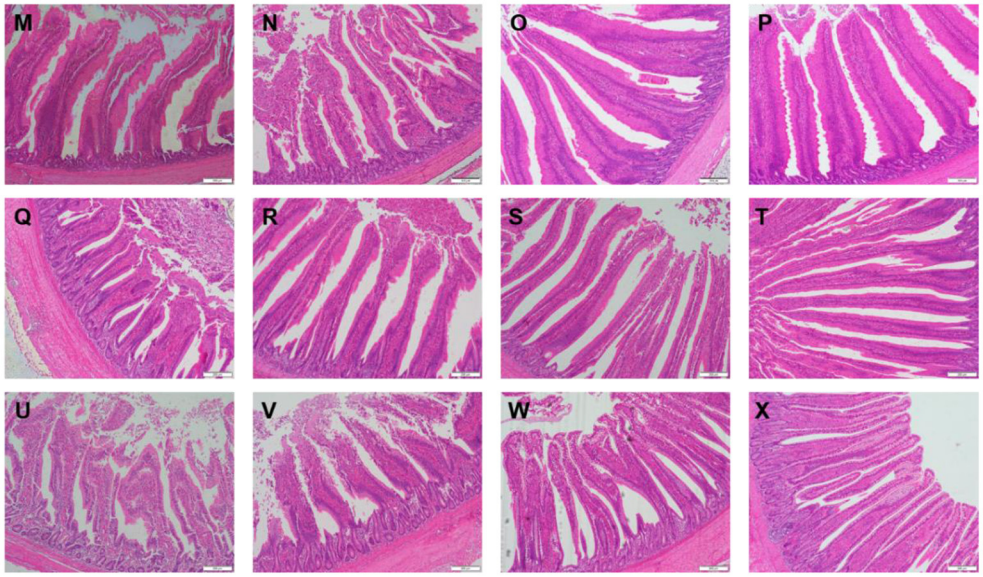

FIGURE 3
Intestinal mucosal histological appearance (hematoxylin and eosin) of the broilers fed diets containing different sources of selenium on D 42. Original magnification 100 ×. Scale bars = 200 μm. **(M–P)** Duodenum histological appearance of the broilers fed CON, SeNa, SeY, and SeCv diets, respectively. **(Q–T)** Jejunum histological appearance of the broilers fed CON, SeNa, SeY, and SeCv diets, respectively. **(U–X)** Ileum histological appearance of the broilers fed CON, SeNa, SeY, and SeCv diets, respectively.

broilers fed SeNa diets. Furthermore, majority of the results in this study showed the similar effects on broilers fed the diets between SeCv and SeY. Therefore, these findings provided scientific experimental bases for improving growth performance and meat quality of broilers with a new choice of Se sources.

TABLE 4 The liver anti-oxidative capacity of the broilers fed diets containing different sources of selenium.

Items	CON	SeNa	SeY	SeCv	SEM	*P*-value
D 21						
T-AOC (mM/g)	0.165^{b}	0.173^{b}	0.182^{b}	0.247^{a}	0.011	<0.001
GSH-PX (U/mg)	20.3^{b}	55.7^{a}	56.8^{a}	56.6^{a}	3.7	<0.001
SOD (U/mg)	129^{b}	148^{a}	151^{a}	159^{a}	3	<0.001
MDA (nmol/mg)	2.13^{a}	1.62^{b}	1.48^{b}	1.60^{b}	0.08	0.012
D 42						
T-AOC (mM/g)	0.479^{b}	0.472^{b}	0.513^{a}	0.524^{a}	0.013	<0.001
GSH-PX (U/mg)	20.8^{b}	48.6^{a}	49.9^{a}	52.9^{a}	2.9	<0.001
SOD (U/mg)	145^{b}	151^{a}	145^{b}	156^{a}	2	0.044
MDA (nmol/mg)	1.72^{b}	2.20^{a}	1.30^{c}	1.17^{c}	0.09	<0.001

$N = 6$ (1 bird per cage). The same letter on the shoulder of the mean in the same line indicates that the difference is insignificant, and the absence of the same letter means that the difference is significant. GSH-PX, glutathione peroxidases; MDA, malondial-dehyde; SEM, standard error of mean; SOD, superoxide dismutase; T-AOC, total antioxidant capacity.

TABLE 5 The meat quality of the breast and thigh muscle in the broilers fed diets containing different sources of selenium.

Items	CON	SeNa	SeY	SeCv	SEM	*P*-value
Breast muscle						
Color						
L*	59.22	61.00	62.78	62.61	2.74	0.623
a*	11.22^{b}	12.25^{ab}	12.32^{ab}	14.65^{a}	0.85	0.012
b*	4.53	4.44	5.14	5.32	0.52	0.674
Drip loss (%)	1.72^{a}	1.38^{ab}	1.04^{ab}	0.80^{b}	0.33	<0.001
Cooking loss (%)	33.46^{a}	31.58^{ab}	29.65^{bc}	27.32^{c}	1.22	0.018
Shear force (N)	32.35^{a}	28.71^{b}	27.89^{b}	26.94^{b}	1.31	0.005
Thigh muscle						
Color						
L*	64.58	63.50	65.85	66.24	2.44	0.910
a*	14.58^{b}	14.26^{b}	15.66^{b}	18.32^{a}	0.85	<0.001
b*	5.76	6.25	6.47	6.25	0.60	0.509
Drip loss (%)	1.16^{a}	1.11^{ab}	0.87^{ab}	0.58^{b}	0.24	0.021
Cooking loss (%)	31.69^{a}	31.89^{a}	30.96^{ab}	28.52^{b}	1.12	0.012
Shear force (N)	15.86	16.36	15.02	15.77	0.77	0.413

$N = 6$ (1 bird per cage). The same letter on the shoulder of the mean in the same line indicates that the difference is insignificant, and the absence of the same letter means that the difference is significant. SEM, standard error of mean.

Se is one of the substantial trace elements for animals. It promotes growth and plays important roles in maintaining normal development and production of animals (20, 21). Generally, the Se concentration in raw feed material is too low to satisfy the needs of animals for development and health. So, Se as feed additives should be supplemented by external sources (22). Se is not only a functional nutrient but also a high toxic mineral element. In China, the limit of Se in mixed feed for poultry is 0.5 mg/kg (23). Se can be simply divided into organic and inorganic sources. Inorganic Se is mainly used in the form of SeNa, which has an economic advantage and is most widely used in animal diets. However, its strong toxicity, low bioavailability, and oxidation potential have adverse effects on animals and the environment (24). Several studies reported that organic Se existed in the form of SeY had good absorption and utilization rates (4, 25). In this current study, the broilers fed SeCv diets showed an improved performance in the earlier stage and similar performance in the whole period compared with the other 2 Se sources diets. Cv is a hyperaccumulating plant newly found in China. It can transfer inorganic Se from the soil into organic Se mainly existed by the form of $SeCys_2$ and MeSeCys. The similar growth performance among these groups demonstrated that SeCv was a well-replacement of other frequently-used Se sources such as SeNa and SeY for broilers.

Intestinal healthy status can be reflected by a series of indicators such as Vh and Cd (26). Vh and Cd are the most intuitive indicators reflecting the morphological and structural integrity of intestinal mucosa (27). Villi are the main components responsible for nutrient absorption in the small intestine, and increased Vh and Vh/Cd can lead to greater absorption of nutrients and improve growth performance (28). In this study, the intestinal Vh and Vh/Cd of broilers fed different sources of Se was improved compared with the broilers fed Se free diets. And the SeCv diets showed the best effects on the intestinal morphology of the broilers among these groups. In agreement with our study, many previous research reported that Se supplementation could improve intestinal Vh and Vh/Cd of animals (29, 30). Se as the key component of several anti-oxidative enzymes, plays a significant role in gut epithelial cell protection from pro-apoptotic oxidant stress, which in turn enhances their growth and development (31). The superiority of SeCv to SeNa may be caused by the capacity of SeNa to bind to the lines of epithelial tissues in the intestinal lumen, thereby being inaccessible for assimilation and transfer to tissues (32).

The antioxidant system of broilers is regulated through many important anti-oxidative enzymes, including GSH-PX, SOD and so on (33). T-AOC reflects the body or organ cumulative effect of all antioxidants (34). MDA is an important marker for reflecting the degree of lipid peroxidation and the extent of cellular damage (35). As the rapid progress of poultry breeding, although the broilers show improved performance, they are easily attacked by external stressors such as high density and anti-nutritional factors which leading to excessive production of reactive oxygen species (36). The antioxidant effect of Se is generally achieved by GSH-PX, because Se is a component of its active center element (37). This study showed the similarly positive effects of SeCv and SeY on liver anti-oxidative capacity of broilers which is superior to the broilers fed SeNa and CON diets. Some research demonstrated that organic Se was more effective than inorganic Se in improving anti-oxidative capacity (38). The reason of similar results of SeCv and SeY may be that the 2 sources of Se were both mainly existed in the form of Selenoprotein (4, 39).

Meat color, drip loss, cooking loss and shear force are all important indicators for reflecting the meat quality (40). There were studies demonstrated that Se could increase the capacity of oxidation resistance, prevent the myoglobin or oxymyoglobin being oxidized to metmyoglobin, deepen the muscle chroma, increase meat redness, and improve meat quality (41, 42). Drip loss and cooking loss reflect the ability of muscle proteins to attract water and hold it within the cells (43). Shear force reflects the tenderness which is associate with the mouthfeel of the meat (44). When the animals were under acute or chronic stress, the above parameters of meat quality usually increased (45). In this study, the broilers fed SeCv diets had higher thigh muscle redness compared with other Se sources diets and similar scores of drip loss, cooking loss and shear force compared with SeY diets. These results were in accordance with the growth performance and anti-oxidative capacity which illustrated that SeCv was a well-additive to improve meat quality of breast and thigh muscle of broilers and had a similar effect to SeY.

Conclusion

In conclusion, the broilers fed SeCv diets had a better effect compared with SeNa and a similar effect compared with SeY on growth performance, anti-oxidative capacity and meat quality. These results recommended that SeCv was a well Se source for broilers diets.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Wuhan, China). The chicks used in the study were obtained from the Xiangyang Charoen Pokphand Co., Ltd. (Xiangyang, China). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XX and YW wrote the manuscript. YZ, XJ, XC, QG, SC, ZZ, HZ, JZ, and YL read and approved the final version. All authors were involved in study design and implementation, data acquisition, analysis, and interpretation.

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Conflict of interest

Authors YZ, XC, and QG were employed by the company Enshi Se-Run Material Engineering Technology Co., Ltd. Enshi, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Emerging roles of selenium on metabolism and type 2 diabetes

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Selenium is recognized as an essential element for human health and enters human body mainly *via* diet. Selenium is a key constituent in selenoproteins, which exert essential biological functions, including antioxidant and anti-inflammatory effects. Several selenoproteins including glutathione peroxidases, selenoprotein P and selenoprotein S are known to play roles in the regulation of type 2 diabetes. Although there is a close association between certain selenoproteins with glucose metabolism or insulin resistance, the relationship between selenium and type 2 diabetes is complex and remains uncertain. Here we review recent advances in the field with an emphasis on roles of selenium on metabolism and type 2 diabetes. Understanding the association between selenium and type 2 diabetes is important for developing clinical practice guidelines, establishing and implementing effective public health policies, and ultimately combating relative health issues.

KEYWORDS

selenium, dietary intake, glucose and lipid metabolism, type 2 diabetes, selenoproteins

Introduction

Selenium is a natural chemical element existing in soil, water, and air. Selenium reaches the human body by food chain through incorporation into plants, animals, and aquatic organisms (1). From the public health perspective, selenium is treated as an essential micronutrient and is commonly used in dietary supplementation products widely consumed in western countries (2, 3). In human beings, the nutritional functions of selenium are achieved by 25 selenoproteins (4), with essential enzymatic functions, including hydroperoxide/phospholipid peroxide reduction (glutathione peroxidases, GPxs) (5, 6), thiol redox status regulation (thioredoxin reductases) (7), thyroid hormone activity regulation (iodothyronine deiodinases, Dios) (8), selenium transport (selenoprotein P, SelP) (9) and some are yet to be determined. Selenium is reported to play a role in maintaining redox balance, anti-cancer, and improving immunity, and is closely related to Keshan disease, diabetes, mental disorder, inflammation, and infections (10, 11).

Diabetes is a highly costly chronic disease (12), with an estimated worldwide prevalence of 463 million adults according to the 2019 report of the International Diabetes Federation Diabetes Atlas (IDF) (13). Type 2 diabetes accounts for 90% of all diabetes and is characterized by defective insulin secretion and/or insulin resistance (14). Although the mechanisms of insulin resistance and type 2 diabetes remain not fully understood, accumulating evidence suggests that oxidative stress plays an important role in both onset and progress (15). As several selenoproteins have the potential to protect the body from oxidative stress, selenium is expected to be protective against type 2 diabetes (10, 16). However, recent evidence raised concerns that a high level of selenium exposure may be associated with an increased risk of type 2 diabetes (17–20).

Even though how selenium affects the risk of type 2 diabetes is conflicting, evidence has confirmed that people with low status may benefit from additional selenium intake (21, 22). Although, selenium is assumed to be helpful in the prevention and therapy of type 2 diabetes (23, 24), selenium intake, including selenium supplementation, should be excluded for primary or secondary diabetes prevention in populations with adequate selenium status (25). This review focuses on the association of dietary selenium with type 2 diabetes epidemiology and discusses the major selenoproteins in the regulation of glucose and lipid metabolism and their implication in the development of type 2 diabetes.

Selenium in food

Diet is a major source of selenium for humans and the content of selenium in foods varies greatly (**Figure 1**). The main source of selenium in diet are cereals because of the large amounts consumed as well as meats and seafood with high protein contents (26). Also, plant-based sources of selenium, including wheat, pearl millet, and maize, are more effective in reversing the deleterious effects of selenium deficiency (27). Natural fruits generally contain low amounts of selenium, rarely exceeding 10 μg/kg and vegetables with a maximum concentration of 6 μg/kg (28). From the selected investigation, the main food sources in diet of selenium intake include cereals, meat products, milk and dairy products, beverages, fish and seafood (29–31). All these groups provide more than 85% of the selenium intake. In addition, ready-to-eat meals, vegetables, fruits, sweets, and beverages contribute to a small part of the dietary selenium intake. Processing technology could affect selenium content in food and bioaccessibility of selenium species, among which soaking, fermentation were reported to increase the bioaccessible selenium content and heating declined the bioaccessibility of SeMet and SeCys (32, 33). Another selenium source of exposure is well-known as selenium supplementation. Considering the low abundance of selenium in daily foods, consuming a diet with natural selenium concentrations is not abundant. Hence, scientific works have dealt with selenium-biofortification strategies to obtain selenium-enriched food or feed, *via* plant cultivation (soil fertilization with inorganic selenium), animal feeding (with selenium-enriched plants), microorganism transformation (fermentation) (34, 35). Asides from the classical methods, pharmacological products and nano-selenium applications can also increase the selenium concentration in the human body (36, 37).

Not only the total intake of dietary selenium but also the selenium species ingested is important to human health (38). Selenium exists in inorganic and organic forms and intakes of different foods are correlated with different types of selenium species. Organic selenium forms in foods mainly contain selenomethionine (SeMet), selenocysteine (SeCys), selenium-methylselenocysteine (SeMeCys), and gamma-glutamyl-Se-methylselenocysteine (GGMSC), and drinking water mainly contains inorganic selenium species such as selenate and selenite (36, 39). Efficient uptake and metabolism of dietary selenium primarily depend on its chemical forms (40). Organic selenium is more easily absorbed by the human body compared with inorganic selenium (41), and more than 90% of SeMet is absorbed in human body but only about 50% of selenium is from selenite. In addition, nano-selenium attracts growing attention due to its high chemical stability, biocompatibility, and low toxicity (42, 43). Nano-selenium has been applied as antioxidants, dietary supplements, antidiabetic agents (37, 44).

Selenium has long been termed "an essential poison" as selenium doses exceeding 400 μg/day may exert toxic actions according to the World Health Organization (WHO) (45). The recommended daily allowance (RDA) of selenium varies hugely depending on the geographical area, ranging from 25 μg/day for adult women in Japan up to 100 μg/day in the Netherlands and Macedonia, but most RDA levels are in the range of 50–60 μg/day. Both selenium deficiency and excess have been associated with adverse health effects, and the health effects of selenium are recognized as the inextricable U-shaped link with status (10). The selenium content in diabetes serum is commonly lower than normal ones, while additional concerns were raised about the diabetes risk associated with selenium intake above the RDA (55 μg/day) (46). Overall, selenium exposure adds to type 2 diabetes risk across a wide range, especially above dietary intake of 80 μg/day and blood selenium of 120 μg/L (17). Whereas people with low selenium status may benefit from additional selenium intake, those with adequate-to-high selenium status might be affected adversely and should not take selenium supplements.

Selenium in metabolism

Selenoproteins

The biological actions of selenium are mainly mediated by selenoproteins. Selenium is integrated into selenoproteins in the form of selenocysteine. To date, 25

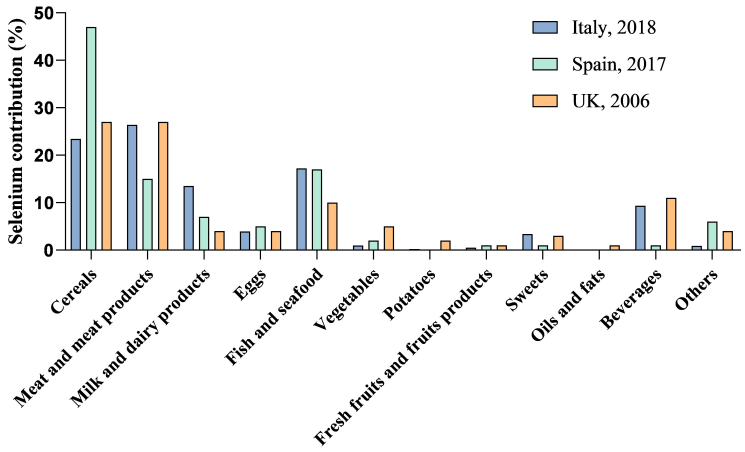

FIGURE 1
Selenium contribution to total dietary selenium intake. Data for selenium contribution to total dietary selenium intake are adapted from published studies. Diet is the main source of selenium for human beings. The species of food, selenium content in food, as well as the amount taken in diet affect the daily selenium intake and selenium status.

selenoproteins have been identified in humans, among which glutathione peroxidase 1 (GPx1), selenoprotein P (SelP), selenoprotein S (SelS), selenoprotein V (SelV), and iodothyronine deiodinases (Dios) have been reported to associate with glucose and lipid homeostasis (**Table 1**) (47, 48).

TABLE 1 Role of main selenoproteins associated with metabolism and type 2 diabetes.

Selenoproteins	Tissue distribution	Cellular location	Physiological function	Health effects on diabetes
Cytosolic glutathione peroxidase 1(GPx1)	Ubiquitous, highly expressed in erythrocytes, liver, kidney, lung	Cytosol and mitochondria	GPx1 reduces intracellular hydrogen peroxide and lipid peroxides	Overquenching intracellular reactive oxygen species, regulating the concentration of hydrogen peroxide (49)
Selenoprotein P (SelP)	Expressed in the liver, heart, brain, and kidney	Secreted into the plasma	SelP functions as a selenium-transporter and maintains selenium homeostasis and possesses antioxidant activity	Promoting insulin resistance (50)
Selenoprotein S (SelS)	Plasma, various tissues	Endoplasmic reticulum (ER) membrane and plasma membrane	SelS promotes ER-associated degradation of errant proteins to increase the translocation of misfolded proteins to the cytosol	Antioxidant protection and anti-ER stress effects in the pancreas (51), up-regulating glucose utilization and down-regulating glucose output in the liver (52) Positively correlates with serum amyloid A in skeletal muscle (53) Positively correlates with HOMA-IR in adipose tissue (54) Negatively correlates with fasting plasma glucose in serum (55)
Selenoprotein V (SelV)	Testis (mainly in seminiferous tubules)	Cytoplasm and nuclei	Regulation of body selenium metabolism and lipid metabolism	Inhibitor of body fat accumulation and activator of energy expenditure (56) Protection against endoplasmic reticulum stress and oxidative injury induced by pro-oxidants (57)
Iodothyronine deiodinases (Dios)	Dio1: liver, kidney, and thyroid Dio3: placenta, brain, gastrointestinal tract, skin, and liver Dio2: pituitary, brain, brown adipose tissue, skeletal muscle, thyroid, heart, and ear	Dio2: endoplasmic reticulum membrane Dio1 and Dio3: the plasma membrane	Thyroid hormone-regulating iodothyronine deodinase	Regulation of energy homeostasis (58)

GPx1 is localized in the cytosol and mitochondria. This enzyme can catalyze the reduction of hydrogen peroxide (H_2O_2) and lipid hydroperoxides using GSH as a reducing cofactor (59). GPx1 is highly sensitive to changes in both selenium status and oxidative stress conditions (60). GPx1 is involved in regulating insulin synthesis and secretion, insulin sensitivity, glucose and lipid homeostasis and the onset and progression of diabetes (61). SelP functions as a selenium-transport protein to deliver selenium from the liver to other tissues to maintain appropriate selenium levels in tissues. Selenium is necessary for the synthesis of antioxidative selenoproteins. Thus, SelP plays an important role in the cellular antioxidative system by maintaining these selenoproteins. Furthermore, SelP possesses multifunctional properties such as GPx-like antioxidant enzyme activity (62), peroxynitrite scavenging (63), and metal-binding activity (64). SelS is resident in the endoplasmic reticulum (ER). It is involved in regulating oxidative stress, ER stress, and inflammatory response (51, 52, 65). SelS plays roles in antioxidant protection and anti-ER stress in the pancreas and blood vessels, while it promotes insulin resistance in the liver, adipose tissue, and skeletal muscle (51, 66). SelV is highly expressed in the testis (mainly in seminiferous tubules). It is involved in regulating body selenium metabolism and lipid metabolism (56, 57). Dios, including Dio1, Dio2, and Dio3, are thyroid hormone-regulating iodothyronine deiodinase. Dios are expressed in multiple tissues. Dio1 and Dio3 are located in the plasma membrane and Dio2 is located in the endoplasmic reticulum membrane. Dios play an important role in thyroid hormone signaling involving many key reactions in energy homeostasis and individual growth and development (58, 67).

Glucose metabolism

Selenoproteins are involved in regulating glucose metabolism. Early studies found that inorganic selenium showed an insulin-like effect. Sodium selenate promoted glucose transport and glucose metabolism through the mitogen-activated protein/myelin basic protein kinases (MAPK) and ribosomal S6 protein kinases in rat adipocytes at very high doses (68–70).

Most selenoproteins are antioxidant enzymes and play roles in maintaining insulin secretion with their antioxidant activity. SelP is relatively highly expressed in pancreatic islets, which acts as an antioxidant to protect β cells (42). GPx1 can degrade intracellular H_2O_2 (59). In pancreatic islets, GPx1 reduces the damage of H_2O_2 on β cells and promotes the normal secretion of insulin.

SelP synthesis is regulated like a gluconeogenic enzyme in the liver. SelP gene expression is regulated by the interaction of the transcription factors FoxO1 and HNF-4α with the co-activator PGC-1α (71, 72). These transcription factors similarly control the expression of gluconeogenic enzymes: G6PC (glucose-6-phosphatase, G6Pase, catalytic subunit) and PCK1 (phosphoenolpyruvate carboxykinase, PEPCK), which are involved in hepatic glucose release to adapt to feeding and fasting (73, 74). It was found that liver SelP mRNA levels have been shown to increase during fasting and decrease after feeding in mice, which indicated that the liver could fine-tune SelP secretion according to the nutritional state (72). Furthermore, SelP transcription can be inhibited by insulin *via* the PI3K/Akt/FoxO1 pathway (72, 75). Insulin-induced phosphorylation of FoxO1 results in its nuclear exclusion and inhibition of FoxO1-dependent transcription of SelP in liver cells (71). Thus, insulin-mediated regulation of hepatic SelP production and secretion represents a physiological link between selenium homeostasis and carbohydrate metabolism (71).

Selenium deficient diet results in impaired islet function, low insulin secretion, and high blood glucose (76, 77). The deficiency of selenium decreases the expression of several selenoprotein genes and proteins in different tissues, which may dysregulate glucose homeostasis. It was reported that GPx1 deficiency induced type 1 diabetes-like phenotype (61). GPx1-knockout-mice developed islets β cell damage and insulin reduction (78, 79). Hepatic-specific deletion of SelS in mice caused obesity, hepatic steatosis, insulin resistance, and disturbed glucose homeostasis (66, 80). It was reported that reduced synthesis of selenoproteins, including GPx1 and MsrB1, caused by overexpression of an i(6)A(-) mutant selenocysteine tRNA promoted glucose intolerance and led to a diabetes-like phenotype (81). Selenocysteine lyase (Scly) is the enzyme that supplies selenium for selenoprotein biosynthesis *via* decomposition of the amino acid selenocysteine (82). Moreover, it was found that Scly knockout mice fed with low selenium dietary reduced GPx1 and SelS protein levels and affected hepatic glucose homeostasis (83). Taken together, selenium and selenoproteins play important roles in glucose metabolism, especially in maintaining a redox balance to promote the normal synthesis and secretion of insulin.

Lipid metabolism

Selenoproteins are also involved in regulating hepatic lipid accumulation. It was reported that SelS expression was down-regulated in the liver in high-fat diet (HFD)-fed mice and db/db mice, and SelS expression levels were reduced in the PA-induced primary hepatocytes (66).

Hepatic triglyceride synthesis consists of fatty acid uptake and *de novo* lipogenesis (84, 85). It was reported that serum free fatty acids level was elevated in hepatocyte-specific SelS knockout (SelSH-KO) mice, and the expression levels of cluster of differentiation 36 (CD36), fatty acid transport protein 2

(FATP2) and fatty acid transport protein 5 (FATP5), which are involved in fatty acid uptake, were markedly increased in the liver of SelSH-KO mice (66). On the other hand, the expression levels of peroxisome proliferator-activated receptor α (PPARα), carnitine palmitoyltransferase 2 (CPT2), and acyl-coenzyme A oxidase 1 (ACOX1), which are responsible for fatty acid oxidation, were down-regulated in SelSH-KO mice. These results suggested that hepatic SelS deletion increased hepatic triglyceride and diacylglycerol accumulation *via* promoting fatty acid uptake and reducing fatty acid oxidation (66).

FGF21 is an endocrine hepatokine produced predominantly in the liver (86, 87). Hepatic-specific deletion of SelS (SelS LKO) decreased the production of hepatokine FGF21 and adipokine adiponectin and increased adipose tissue size. These results indicated that the FGF21-adiponectin axis was inhibited in SelS LKO mice, which exacerbated hepatic metabolic disorders (66). Taken together, these studies show that selenoproteins participate in regulating lipid metabolism, especially in lipid intake and fatty acid oxidation, which indicates that selenoproteins may be a potential intervention target for lipid metabolic disorders.

Epidemiology of selenium and type 2 diabetes

Selenium is expected to protect against type 2 diabetes because of the potential of several selenoproteins to protect against oxidative stress (19, 88, 89). Selenium intake varies greatly among countries due to the selenium differences in local soil and foods consumed (**Figure 2**). The relationship between selenium level and the prevalence of type 2 diabetes is possibly U-shaped, with possible adverse effects occurring both below and above the physiological range for optimal activity of some or all selenoproteins (90). Whereas dietary selenium supplement has been applied to improve glucose metabolism, accumulating evidence showed that exposure to a high level of selenium increased the risk of type 2 diabetes. In a multivariate logistic regression model, an increase of 10 μg/L in selenium induced to the prevalence of diabetes mellitus by 12% (91), showing a dose-dependent relationship between selenium level and diabetes. Higher serum selenium was discovered to be linked with increased plasma glucose levels and glycosylated hemoglobin levels (92). Intake of high-level selenium might affect the expression and(or) function of key regulators for glycolysis, gluconeogenesis, and lipogenesis (20). Furthermore, the association between selenium and type 2 diabetes was independent of insulin resistance at high serum selenium levels (19, 91). The prevalence of diabetes, as well as glucose and glycosylated hemoglobin levels, increased with increasing selenium concentrations up to 140 μg/L of selenium exposure (93), while several studies insisted on a risk serum selenium level of 160 μg/L (17, 94). In addition, the non-experimental studies reached agreement with the findings from randomized controlled

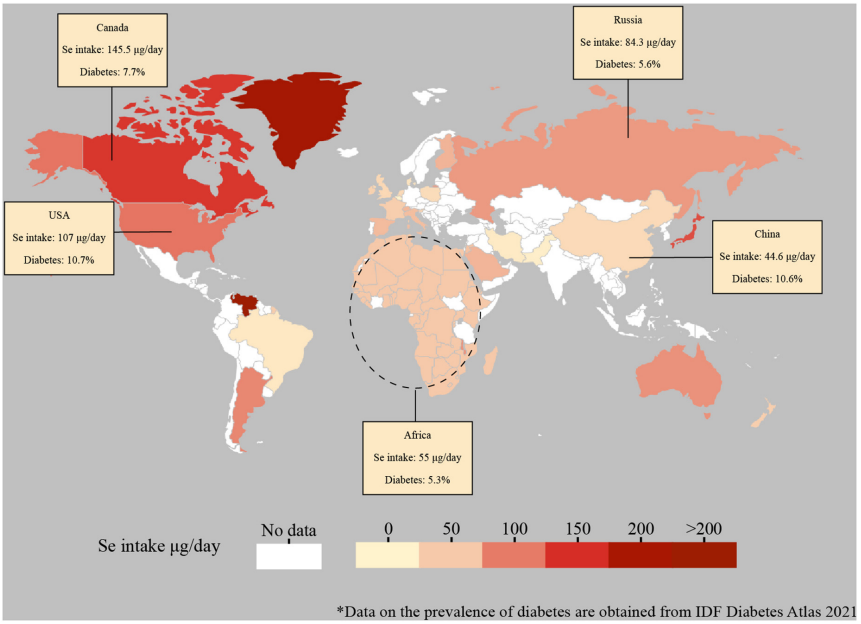

FIGURE 2
Global estimated daily intake of selenium and prevalence of diabetes. Data for the global estimated daily selenium intake are adapted from the published studies (95-102). The selenium data of mainland China was selected. The selenium data of Africa was adapted from the Africa selenium daily supplied amount. Data for the prevalence of diabetes (data included until 2021) are derived from the Diabetes Atlas of the International Diabetes Federation (https://diabetesatlas.org/data/en/indicators/2/). Dashed lines are not supposed to accurately represent regions.

trials, illustrated that selenium exposure from moderate to high levels is associated with increased risk for type 2 diabetes. Further research is required to clarify the optimal range of selenium intake and status for minimizing the potential adverse effects on glucose metabolism and preventing type 2 diabetes.

Mechanisms of selenium in type 2 diabetes

Type 2 diabetes is characterized by defective insulin secretion and/or insulin resistance, and the potential molecular mechanisms include interference of oxidative stress, insulin signaling, gluconeogenesis, and endoplasmic reticulum (ER) stress (**Figure 3**). Abnormal selenium status affects the occurrence and development of diabetes through these mechanisms.

β cells and pancreas

Pancreas β cells have a fragile antioxidant system. The expression levels of antioxidant enzymes in pancreatic islets are substantially lower compared with various other tissues, which renders β cells sensitive toward oxidative and nitrosative stress (103). Severe antioxidant selenoproteins deficiency may result in oxidative damage of β cells and lower insulin secretion. GPx1-knockout-mice fed a high-fat diet for 12 weeks decreased plasma insulin and glucose-induced insulin secretion (78, 79). It was reported that GPx1-knockout-mice elevated islet superoxide and hydroperoxide production and up-regulated p53 phosphorylation. By contrast, after overexpressing GPx1 in pancreatic β cells, C57BLKS/J mice were protected from the β cell damage when stimulated with streptozotocin, and db/db mice exhibited reversed hypoinsulinemia and hyperglycemia (104). However, global overexpression of GPx1 induced obesity, hyperglycemia, insulin resistance in mice, and developed type 2 diabetes-like phenotypes (105–107), which infered adverse effects of excessive selenoprotein biosynthesis and the complexity of redox status. The results of SelP further prove this point. It has been shown that excess SelP impairs the function of pancreatic β cells and decreases insulin secretion (50, 108). The injection of purified human SelP protein in mice resulted in a decrease in insulin levels, a decline of β cells and α-cells in the pancreas, and also a rearrangement of the position of these cells in the pancreatic islets (50). Furthermore, the administration of SelP-neutralizing antibodies could improve insulin secretion and glucose intolerance in a mouse model of diabetes (50). Thus, selenium homeostasis and redox balance are extremely important for β cells and insulin secretion.

Insulin signaling

The antioxidant activity of selenoproteins can protect the islets from oxidative stress, but excessive antioxidant activity is not beneficial for insulin signaling. The binding of insulin to its receptor activates NADPH oxidase enzymes and results in the production of space H_2O_2 (109). These small amounts space of H_2O_2 can act as second messengers and are required to deactivate two insulin-signaling inhibitors: tyrosine phosphatase 1B (PTP-1B) and phosphatase and tensin homolog protein (PTEN). This H_2O_2-mediated deactivation is considered to enhance the insulin-induced PI3K/Akt signaling, which facilitates glucose uptake, inhibits glycogen synthesis, and suppresses gluconeogenesis (110). GPx1 can degrade intracellular H_2O_2 and regulate its concentration (59). However, when intracellular physiological H_2O_2 is eliminated by excessive activity of GPx1, insulin signaling may be impaired. In this regard, GPx1-knockout-mice were protected from insulin resistance induced by a high-fat diet due to increased H_2O_2 production and inactivation of PTEN (111). Conversely, mice over-expressing GPx1 exhibited insulin resistance and hyperinsulinemia (105–107). Therefore, the appropriate amount of selenium and selenoproteins will benefit insulin function. Based on the control of SelP transcription through PGC-1α/FoxO1/HNF-4α, it was found insulin could inhibit SelP transcription by the PI3K/Akt/FoxO1 pathway (75). High levels of SelP impaired insulin signaling and dysregulated glucose metabolism both in the liver and muscle *via* the inactivation of adenosine monophosphate-activated protein kinase (AMPK) (72, 112). So, SelP has been identified as a "hepatokine" that induces insulin resistance and excess SelP promotes type 2 diabetes (50, 108). Thus, a high level of selenoproteins may impair insulin sensitivity through the interference of the insulin signaling cascade.

Gluconeogenesis

Plasma SelP levels were reported elevated in patients with type 2 diabetes, and there was an association between high plasma selenium and fasting plasma glucose in type 2 diabetes patients (47, 113, 114). SelP and gluconeogenic enzyme gene expression are similarly regulated by methylation of the same transcription factors (71, 72). SelP, together with G6PC and PCK1 is transcribed through PGC-1α/FoxO1/HNF-4α. Under the normal metabolic condition, insulin inactivates the transcription of SelP and gluconeogenic enzymes. Under the condition of high glucose and insulin resistance, the dysregulated transcriptional activity of FoxO1 enhances the biosynthesis of SelP and gluconeogenic enzymes, which results in elevated plasma SelP and selenium levels and further elevated plasma glucose levels (72, 115, 116). Thus, from this

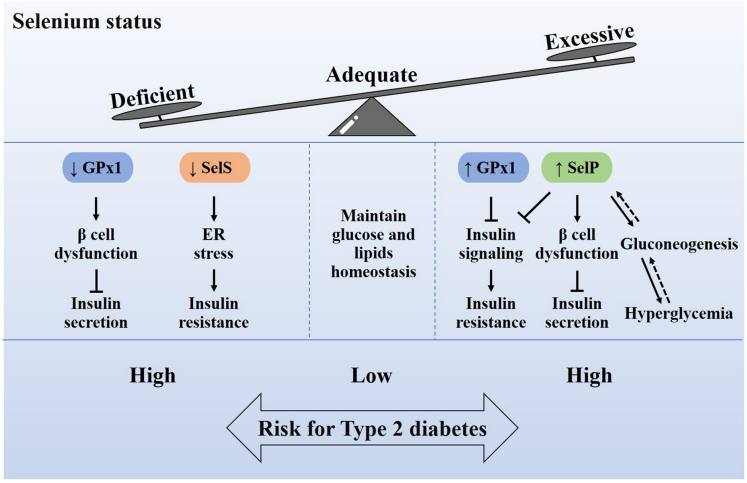

FIGURE 3
Relationship between selenium homeostasis and type 2 diabetes. Adequate selenium intake is very important for maintaining the homeostasis of glucose and lipid metabolism. Excessive or insufficient selenium intake will cause the increase or decrease of selenoproteins in the body, which in turn leads to a high risk of type 2 diabetes. The abnormal content of selenoproteins, including Gpx1, SelP, and SelS, may cause oxidative damage of β cells, insulin signaling impairment, endoplasmic reticulum stress and gluconeogenesis, which induce insulin secretion defects or insulin resistance. Therefore, it is recommended to supplement selenium according to the state of selenium.

point, elevated plasma SelP levels might be considered as the result rather than the cause of hyperglycemia and insulin resistance (117).

Endoplasmic reticulum stress

Chronic endoplasmic reticulum (ER) stress affects glucolipid metabolism, which is crucial to the occurrence and development of insulin resistance and nonalcoholic fatty liver disease (NAFLD) (118, 119). ER stress is induced by unfolded or misfolded proteins accumulated in the ER, which initiates unfolded protein response (UPR) to restore homeostasis in the ER. ER-associated protein degradation (ERAD) is activated to remove unfolded or misfolded proteins (120). Selenoproteins, such as SelS and SelK, are induced under ER stress, which play important roles in ERAD and ER stress. Mechanistically, SelS forms a multiprotein complex with degradation in endoplasmic reticulum protein 1 (Derlin1)-ubiquitin ligase E3-p97ATPase and SelK to participate in ERAD, which mediates misfolded proteins in the ER to be translocated back into the cytosol for degradation by the proteasome (121–123). Consistently, knockdown of SelS increased the expression of ER stress marker genes (124, 125), whereas its overexpression protected against ER stress injury in hepatocytes and cell lines (65, 66, 125, 126). Consistently, ER stress was increased in SelS-hepatic-knockout mice and SelS-knockdown hepatocytes, but suppressed in SelS-overexpress hepatocytes (66). It was likely that excessive misfolded or unfolded proteins were accumulated in SelS deficiency hepatocytes due to impaired ERAD capability, resulting in chronic ER stress (66). Collectively, these results show evidence supporting that SelS has the potential to reduce ER stress injury and may protect hepatocytes from the development of insulin resistance and hepatic steatosis.

Conclusion and future perspectives

In conclusion, we systemically review the role of selenium and selenoproteins in type 2 diabetes and indicate the therapeutic potential of selenium supplementation in the treatment of metabolic disorders. Even though the interaction of some other selenoproteins with type 2 diabetes has not been verified, their effective roles in the regulation of glucose and lipid metabolism are becoming increasingly clear. Although there have been some inconsistent results, extensive evidence has suggested that selenium supplementation is beneficial for preventing and treating several chronic diseases (127). Future studies are needed to explore the association between selenium exposure and metabolic effects in more details with selenium exposure, and the potential mechanisms.

Selenium supply is very important for maintaining glucose and lipid homeostasis in healthy adults and patients with type 2 diabetes. However, the epidemiology of observational and experimental studies of selenium in type 2 diabetes reveal that both selenium deficiency and severe excess lead to insulin resistance and β cell dysfunction, with potential molecular mechanisms including interference of oxidative stress, insulin signaling, gluconeogenesis, and ER stress. Thus, selenium should be supplemented according to the status of selenium, while excessive selenium supplement is not recommended.

Nutrigenetic research has identified several single nucleotide polymorphisms in selenoproteins, which may clarify the high variability of selenium nutritional status in different populations (34, 43) and influence metabolic parameters in response to selenium supplementation (44). Thus, more personalized nutritional recommendations are needed to consider not only the regional particularities but also the genetic characteristics of the population or individuals.

Author contributions

JZ, HZ, and YL researched data for the manuscript, made substantial contributions to discussions of the content, and wrote the manuscript. YH and XW edited the manuscript with important intellectual content. All authors reviewed and edited the manuscript before submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Advances in the study of selenium and human intestinal bacteria

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Selenium (Se) is an essential trace element for humans and has conveyed great a wide range of interests due to its contribution to health. Presently, the regulatory mechanisms of selenium on human health, especially the regulatory mechanisms of selenium on human intestinal (gut) microflora and its effects on diseases are receiving attention from academic circles. This review involves the effects of selenium on physical health, the relationship between selenium and intestinal microflora, and the progress of research between selenium, intestinal microflora, and diseases. Furthermore, the current status of research on the selenium, intestinal microflora, and diseases is also presented.

KEYWORDS

selenium, human health, intestinal bacteria, microflora, disease

1 Introduction

The intestinal tract is the second "brain", of human beings and is vital to human health. It is an important place for the absorption of nutrients and excretion of metabolic wastes in the body and contains more than 400 species of intestinal bacteria, which play an essential role in nutrient absorption, participating in body metabolism, and immune enhancement. The balance of intestinal microflora is very important to maintain human health. When the intestinal microflora is disturbed and out of balance, it can easily lead to a series of diseases, such as cancer, diarrhea, autism, and enteritis (1). Colorectal cancer is the second leading cause of cancer deaths in Europe, and in 2015 scholars from 10 European countries investigated the relationship between selenium and colorectal cancer incidence in up to 520,000 subjects, Results indicate that selenium levels is associated with the perceived level of colorectal cancer risk, which increased selenium intake in low selenium regions may decrease the risk as a result (2, 3). Meanwhile, experts from Penn State University found that selenium levels in within individuals were strongly associated with the development of inflammatory bowel disease, and that lower selenium levels were associated with greater susceptibility to inflammatory bowel disease.

This is because selenium can be involved in regulating the intestinal inflammatory response, with changes in cellular oxidative status coupled with altered selenoprotein expression in macrophages driving the switch from a pro-inflammatory to an anti-inflammatory phenotype, resulting in the elimination of intestinal inflammation and restoration of the barrier role of the intestinal epithelium (4).

Se can be divided into organic and inorganic selenium. Cereals, meat, fish, and nuts are the best sources of Se. Among these, Brazil nuts, are one of the foods with the highest Se content, ranging from 1.80 to 320.80 μg Se/g (5). In addition, selenium intake is also regionally related, and studies have found that in areas with low soil selenium content, selenium levels in drinking water and crops are generally low, resulting in inadequate selenium intake by the local population (6). In China, 72% of the soil environment is deficient in selenium, which cannot meet the human body's demand for selenium (7). Therefore, scientific selenium supplementation is very important, usually you can supplement selenium-rich foods, in the case of serious selenium deficiency, you should supplement selenium-containing preparations. Sulfur amino acid analogs are organic forms of Se, including selenomethionine (SeMet), selenocysteine (SeCys), and methylated derivatives. The inorganic forms are selenates, such as selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}). Indeed, the bioavailability of selenium depends mainly on its chemical form. Under normal conditions, organic selenium is absorbed more rapidly and is usually used in the biosynthesis of selenoproteins (8). The organic form of selenium (selenomethionine) has higher bioavailability and lower toxicity than its inorganic form (sodium selenite). In addition, selenium nanoparticles prepared from sodium selenite, ascorbic acid and chitosan, added to soy sauce, can enhance the antioxidant activity of soy sauce (9). Studies have shown that the biosynthesis of organic selenium can be accomplished by whole-cell biotransformation using sodium selenite under controlled *Bifidobacterium bifidum* BGN4 culture conditions, with a maximum organic selenium content of 207.5 μg/g in *Bifidobacterium selenium*-enriched BGN4 (10).

Organic or inorganic selenium is absorbed in the intestine and subsequently transported to the liver, where it is metabolized and produces selenoproteins, which are subsequently transported to other tissue sites in the body, thereby having an impact on human health. In addition, several lactic acid bacteria can maintain the sodium selenite state in the form of SeCys and SeMet in cells, thus providing a bioavailable form of Se, which is usually responsible for the poor absorption of inorganic selenium in human cells (11). The amount of Se intake has been reported to affect the barrier function and immune response in the intestine. Selenium is essential for maintaining the immune system, conversion of thyroid hormones, and reducing the risk of chronic diseases (12). In addition to this, selenium can balance intestinal microecology and avoid health damage caused by its imbalance. For example, under lead exposure, the gut experiences reduced microbiota diversity and oxidative stress, and the selenium-rich *Lactobacillus rhamnosus* SHA113 can effectively protect the gut from lead damage by forming an insoluble mixture with lead, which greatly facilitates the efficiency of lead excretion through the feces (13).

2 Selenium and human health

Selenium is one of the most important trace elements in the human body and has significant anti-inflammatory and antioxidant properties that exert antioxidant effects and protect the body from oxidative damage. Both deficiency and excess selenium are associated with the development of certain diseases. Insufficient serum levels of selenium may cause various diseases such as cardiovascular diseases (14, 15). On the other hand, excess selenium may lead to selenosis, which can cause symptoms such as fatigue, tachycardia, and diarrhea (16). Chronic selenium overdose can also lead to liver and kidney necrosis, neurological disorders, and damage to the reproductive and immune systems (5). A study of more than 13,000 followers over 12 years found that serum Se levels ≥135 μg/L was associated with reduced cancer mortality (17). Similarly, a systematic evaluation including 13 meta-analyses showed that high selenium levels were associated with lower morbidity and mortality from cardiovascular disease (18). In another study of over 40,000 randomized trial participants, selenium supplementation was found to reduce serum C-reactive protein levels and increase GPX levels, suggesting a positive effect of selenium on reducing inflammation and oxidative stress in cardiovascular disease (19).

In addition, Se and selenoproteins may play an important role in signaling pathways involved in the pathogenesis of certain diseases, such as IBD and cancer (20). Se deficiency and selenoprotein underexpression also impair innate and adaptive immune responses, and in the colon, Se deficiency and selenoprotein underexpression lead to an increase in inflammatory cytokines (21). Additionally, a low intake of Se may lead to a phenotype of gut microbiota more susceptible to colitis and *Salmonella typhi* infections (22). On the other hand, adequate or high levels of Se diet may optimize the intestinal microflora to prevent intestinal dysfunction and chronic diseases (22). Currently, there are more studies on the relationship between selenium and disease and fewer studies on selenium and intestinal microflora and selenium-intestinal microflora-human health.

3 Selenium and intestinal microflora

Approximately 100 trillion microorganisms, including bacteria, viruses, fungi, and protozoa, live in the human gut (23, 24). Gut microbes are capable of encoding more than

three million genes and perform a range of metabolic processes that are impossible for humans, including the synthesis of vitamins and bioactive compounds, the synthesis of essential and non-essential amino acids, and the metabolism of indigestible carbohydrates, among other functions. In addition, intestinal bacteria play a role in nutrient absorption and act as a barrier to block pathogens. In this process, an important role is played by selenium, one of the essential elements responsible for DNA replication and transcription, and a key cofactor for antioxidant and cellular respiratory bacterial enzymes (25). For example, selenocysteine synthase (SelA) is a pyridoxal phosphate-dependent enzyme that catalyzes the formation of selenocysteine-tRNA in bacteria (26).

Selenium intake in food also affects the selenium status and expression of selenoproteins in the host. Intestinal microflora can use ingested Se to express their selenoproteins. Selenium binding protein 1 is involved in selenium metabolism and redox control and has been identified as a circulating biomarker of cardiac events in patients with the suspected acute coronary syndrome (27). Selenium also affects the composition and colonization of the intestinal microbiota and can have an impact on microflora diversity and composition (28). Dietary selenium can influence the colonization of gut microbes, which in turn can affect host selenium status and selenoprotein expression. Studies have shown that about 1/4 of bacteria have genes encoding selenoproteins, and some of them, such as *Escherichia coli, Clostridium difficile*, and *Enterobacteriaceae*, can colonize the human gastrointestinal tract (29).

The microbiota can influence selenium status, and although the human body and the gut microflora benefit from a symbiotic relationship, the two may compete for selenium when micronutrient (such as selenium) levels become limited (30). On the one hand, the gut microbiota promotes the biotransformation of selenium compounds. Besides, the uptake of selenoproteins by gut bacteria negatively affects the expression of selenoproteins in the host, but the adverse consequences of such effect on human health have not been demonstrated. Se metabolism in humans should be studied to assess whether to recommend an increase in Se intake (29). Furthermore, the effect of probiotics on Se is related to the composition and metabolism of the intestinal microbiota. Se is required for the conversion of T4–T3, and the gut microbiota can interact with Se to express its selenoproteins. Moreover, certain species of gut microbes can increase the bioavailability of selenium and prevent the toxicity produced by its excess (5). Some intestinal bacteria can prevent infection by selenium-dependent bacteria by competing for selenium or by producing metabolites that may be harmful to pathogenic bacteria. In the face of this type of bacterial infection, a complex interaction occurs between the host's immune response, the microbial pathogen, the microbiota, and the Se status of the host. Bacteria with Se-dependent enzymes can survive in the anaerobic conditions of the mammalian gut and these bacteria benefit from the host by using Se to increase their virulence and pathogenicity (31). It was shown that SeNPs with biomolecular shells synthesized by the probiotic bacterium *Lactobacillus casei* 393 have significant antioxidant and anticancer activities and could provide a better option for the synthesis of the biogenic element selenium with potential applications as anticancer and antioxidant agents (32).

It has been shown that anaerobic Trichoderma and Clostridium, particularly *Bacillus fumigatus*, are selenoprotein-rich prokaryotes. Evolutionary trends in Se and selenoprotein use suggest that there are more than 5,200 bacterial genomes, most of which are host-related, of which 2/3 do not use Se, indicating that this capacity has been lost over time (5, 33).

4 Selenium, intestinal bacteria and disease

A lack of Se can reduce an individual's immunity, allowing bacteria that do not require Se to survive to cause infection and lead to disease. The human gut microflora may also differ in the presence of Se, which can disrupt the balance of the human gut microflora by competing for Se or producing metabolites that may be harmful to disease-causing bacteria in order to prevent infection by Se-dependent bacteria (31). Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are characterized by an imbalance in intestinal microbial ecology that leads to altered intestinal dynamics and secretion, allowing for visceral allergy and failure of gut-brain communication (34, 35). Selenium deficiency is common in IBD patients, up to 30.9% (36). Studies have shown that Se is important in improving IBD due to the ability of selenoproteins to reduce the inflammatory response (4). Furthermore, dietary Se content was positively correlated with the presence of Firmicutes and negatively correlated with warty colitis in IBD patients (37).

In addition to its effects on inflammatory bowel disease, selenium can also influence the gut microbiota to have an impact on thyroid function (38). The thyroid gland contains the highest levels of Se and several proteins involved in thyroid metabolism contain Se. The microbiota affects Se uptake and can alter the availability of L-thyroxine and the toxicity of propylthiouracil (PTU). In the presence of normal Se levels, the thyrotropin reductase system and SH-Px protect thyroid cells from damage by peroxides. A decrease in the number of lactic acid bacteria in patients with thyroid disease could confer the bioavailability of Se and its role in activating thyroid hormone conversion (39).

Se prevents oxidative damage during the synthesis of other hormones. In addition, the relationship between selenium and cancer affecting intestinal microflora cannot be ignored. Bacteria of the genus *Dorea* sp. are the most common species in the intestinal microbiota and their abundance is increased under Se deficient conditions, disrupting the homeostatic balance of the gut and affecting host metabolism and immunity, with consequent potential for diseases such as cancer, multiple sclerosis, and non-alcoholic liver disease

(22, 40). Selenium deficiency and inadequate selenoprotein expression also impair innate and adaptive immune responses with high levels of inflammatory cytokines, especially at the colonic level. The impact of the gut microbiota on selenoproteins and other molecules related to redox homeostasis may have implications for the regulation of oxidative stress, apoptosis, inflammation, and immune responses, which appear to have a direct impact on cancer risk and development (41). In a human study, co-supplementation with probiotics containing *Lactobacillus acidophilus*, *B. bifidum, Bifidobacterium longum*, and selenium led to improvements in cognitive function and increased metabolic levels (42). Moreover, probiotics can reduce inflammatory factors and oxidative damage by producing short-chain fatty acids and reducing the production of free radicals in the intestine (5). Considering that selenium uptake by the intestinal microbiota occurs under unbalanced conditions, it may negatively affect the Se supply to the host, thus inducing cancer and intestinal dysfunction. The deficiency of selenoproteins and molecules related to redox homeostasis leads to a gut microbiota phenotype more susceptible to colitis, pathogenic infections, and cancer (22). Lower expression of different selenoproteins was found in colorectal adenoma and cancer tissues, while higher SELENOP concentrations were negatively associated with colorectal cancer risk (2). *Lactobacillus* is an important genus of bacteria in the human gut, capable of increasing Se concentrations in human cells, and the relative abundance of *S.butyricum* ($P < 0.001$) and significantly lower *Lactobacillus* ($P < 0.001$) was observed in thyroid adenomas (43).

5 Expectation

In conclusion, selenium deficiency may lead to phenotypic alterations in the gut microbiota, making humans more susceptible to the development of inflammatory bowel disease, thyroid disease, and cancer, among others. Despite some progress in research between selenium and gut bacteria, many questions remain to be addressed, such as what is the optimal selenium level for healthy gut microbiota? The link between gut microbiota, selenium status, and disease is still difficult to determine, and the complex interactions between microbiota, diet, and the human body may involve multiple mechanisms. Therefore, to address the above questions, a three-pronged approach should be taken.

1) Intestinal bacteria can absorb selenium from the human body, and the relationship between the optimal daily selenium intake in humans and the selenium requirement of normal intestinal microflora needs to be addressed. Selenium is mostly present in the human body in the form of selenocysteine, and the effects and differences of other forms of selenium on the balance of human intestinal microflora are worth exploring.
2) Although some clinical studies have revealed an association between gut microbiota composition and disease development, few studies have provided evidence for the direct role of Se in the gut microbiota. Most studies on selenium and gut bacteria have focused on mice, few have been conducted on humans. Therefore, the Se-gut microbiota-disease relationship needs to be explored in humans, and more clinical studies are recommended.
3) The vast majority of intestinal microflora is not culturable, which poses some difficulties for selenium-gut microflora-disease studies. Based on high-throughput sequencing and the multi-omics combination can exactly bridge the above shortcomings.

Author contributions

JC and HZ conceived and wrote the first draft of the manuscript. JC, WS, XC, and HZ revised each part of the manuscript in detail. All authors participated in the revision of the manuscript, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Trends and recent progresses of selenium nanoparticles as novel autophagy regulators for therapeutic development

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Autophagy, one of the major intracellular degradation systems, plays an important role in maintaining normal cellular physiological functions and protecting organisms from different diseases. Selenium (Se), an essential trace element, is involved in many metabolic regulatory signaling events and plays a key role in human health. In recent years, selenium nanoparticles (Se NPs) have attracted increasing attentions in biomedical field due to their low toxicity, high bioavailability and high bioactivity. Taking the advantage of their advanced biological activities, Se NPs can be used alone as potential therapeutic agents, or combine with other agents and served as carriers for the development of novel therapeutics. More interestingly, Se NPs have been widely reported to affect autophagy signaling, which therefor allow Se NPs to be used as potential therapeutic agents against different diseases. Here, this review suggested the relationships between Se and autophagy, followed by the trends and recent progresses of Se NPs for autophagy regulation in different diseased conditions. More importantly, this work discussed the roles and potential mechanisms of Se NPs in autophagy regulating, which might enhance our understanding about how Se NPs regulate autophagy for potential disease treatment. This work is expected to promote the potential application of Se NPs as novel autophagy regulators, which might benefit the development of novel autophagy associated therapeutics.

KEYWORDS

selenium, selenium nanoparticles, autophagy, regulators, therapeutics

Introduction

Autophagy, an essential and conserved catabolic process, is one of the major intracellular degradation systems that delivers cytoplasmic or invasive materials into autolysosomes for degradation (1). Although there are three typical classes of autophagy (macroautophagy, microautophagy, and chaperone-mediated autophagy), macroautophagy is thought to be the major type of autophagy and is always referred to as "autophagy" (1). Autophagy begins with an isolation membrane, also known as a phagophore to sequester the cargos, such as proteins, organelles, ribosomes and pathogens, in a double-membrane autophagosome (2). Autophagosome matures through fusion with the lysosome to form autolysosomes, which

promotes the degradation of the cargos. Lysosomal permeases and transporters could export amino acids and other by-products of degradation back out to the cytoplasm, where they can be re-used for building macromolecules and for the metabolic uses (2). Thus, autophagy is considered to be a cellular recycling factor that promotes energy efficiency and re-uses the non-functional proteins and organelles (3). Dysfunction of autophagy could induce ubiquitination inhibition, reactive oxygen species (ROS) accumulation, mitochondrial disruption and genomic instability, thus leading to lots of diseased conditions, such as cancer, neurodegeneration, infection, and aging (4, 5). And increasing evidences are suggesting that autophagy can serve an adaptive role to protect organisms against different pathologies, which therefore highlights autophagy as a critical signaling event for novel therapeutic strategy development (6, 7).

Selenium (Se), an essential trace element, has been linked to many health benefits in humans and other mammals, such as decreasing the incidence of cancer (8), protecting against cardiovascular diseases (9), treating particular muscle disorders (10), delaying the onset of AIDS in HIV-positive patients and boosting immune function in mammalian development (11, 12). The biological functions of the micronutrient selenium are mediated in large part by selenoproteins, which contain selenocysteine (Sec) in their active site. Glutathione peroxidase 1 (GPx1), the most abundant selenoprotein in mammals, has been considered as one of the major antioxidant enzymes to protect cells from oxidative damage by degrading toxic H_2O_2 (13). The iodothyronine deiodinase family of selenoproteins is involved in the regulation of thyroid hormone activity by reductive deiodination (14, 15). Another kind of selenoproteins-thioredoxin reductases (TRs) are involved in the control of antioxidant defense and regulation of transcription factors (15), which therefore play critical roles in cancer development and treatment (16, 17). Selenophosphate synthetase is a selenoenzyme that catalyzes the ATP-dependent synthesis of selenophosphate (12). Methionine-R-sulfoxide Reductase B1 (MsrB1), a zinc-containing selenoprotein, can reduce oxidized methionine residues to repair oxidatively damaged proteins (18). These important functions of selenoproteins, especially for their regulation effects in cellular oxidative stress, highlight the important physiological roles of Se in human health and diseased conditions.

Cell death is an irreversible cessation of life phenomena and the end of life. According to the definition of morphological criteria, cell death is always divided into apoptosis, necrosis, autophagy, ferroptosis, and cell scorching, etc. (19). Se is an essential trace element that also plays crucial role in cell death. Selenium deficiency has been widely reported to result in the dysfunction of cellular metabolism, which would lead to different modes of cell death (20, 21). Selenoproteins have also been proved to regulate autophagy in different conditions, which therefore can be served as a kind of protective agent to inhibit dysfunctioned autophagy (22, 23). Moreover, selenium compounds showed strong ability to promote cell autophagy of infected cells to kill the intracellular pathogens (24, 25), which indicated the potential of selenium compounds to serve as anti-infectious agents by promoting autophagy. Selenium compounds also possessed chemotherapeutic effects against multiple malignant cancers by regulating autophagy (26), which highlighted selenium compounds as a new generation of anticancer agents by regulating autophagy.

Nanotechnology has been developed rapidly in the past few decades, which brings novel possibilities for disease treatment. Selenium nanoparticles (Se NPs) have attracted extensive attentions for the application of selenium-based products due to their advantages, including higher safety, lower toxicity, higher bioavailability and stronger free radical scavenging ability and antioxidant activities compared to selenium element (27–30). Zhang et al. (31) reported that encapsulation of selenium in chitosan nanoparticles could improve selenium availability and protect cells from selenium-induced DNA damage response, which indicated the higher safety, lower toxicity, higher bioavailability of Se NPs compared the organic and inorganic selenium compounds. Chen et al. (30) demonstrated the higher bioavailability of Se NPs, and moreover, they also found that Se NPs showed stronger antioxidant activities compared with selenium compounds. Additionally, Se NPs have also been reported to show similar or enhanced activities against diseased cells than Se compounds, but show reduced cytotoxicity against normal cells (32).

The above advantages thus allow Se NPs to be used as a kind of novel nanosystems or selenium products for disease treatment in different conditions. Firstly, Se NPs can also be used as carriers of drugs or other biomolecules for targeted delivery. By encapsulating drugs/biomolecules into Se NPs, the obtained nanosystem could lead to the increased stability and prolonged circulation (33), which therefore provide the enhanced efficiency with better targeting effects. And Se NPs can be easily designed to suit their needs in terms of size, surface charge, genetic, drug loading and controlled release, which is significant for the construction of novel nanomedicines (34–36). Secondly, Se NPs have excellent antibacterial activity and anti-viral activity, which therefore can be used as anti-infectious agents (37, 38). Thirdly, Se NPs also have superior antitumor activities beyond the conventional inorganic and organic selenium compounds, which allows Se NPs to kill tumor cells directly or enhance the anti-cancer activity of current chemotherapeutics (33, 39). Moreover, Se NPs have also been reported to show immunological regulation effects in immune cells (40). And the regulation of autophagy was closely associated with these biological activities for Se NPs. Increasing evidences are suggesting that Se NPs play an important role in the treatment of cancer and infectious diseases through the regulation of autophagy for more effective tumor cell killing and pathogen clearance.

Due to the critical roles of Se NPs in autophagy regulation, this review summarized the effects and mechanisms of autophagy regulated by Se and Se NPs, especially the recent progresses of Se NPs in the treatment of different diseases by regulating autophagy, which is expected to promote the development of novel therapeutics.

Association between selenium and autophagy

The trace element Se can regulate cellular autophagy through different signaling pathways, which is essential for human health. By regulating autophagy under different conditions, selenium compounds and selenoproteins exhibit important effects to induce cancer cell death, reduce drug toxicity, regulate inflammatory responses and resist pathogenic bacterial infections. Considering the critical roles of Se in autophagy regulation, we summarized the mechanisms Se regulates cellular autophagy through various signaling pathways (**Figure 1**).

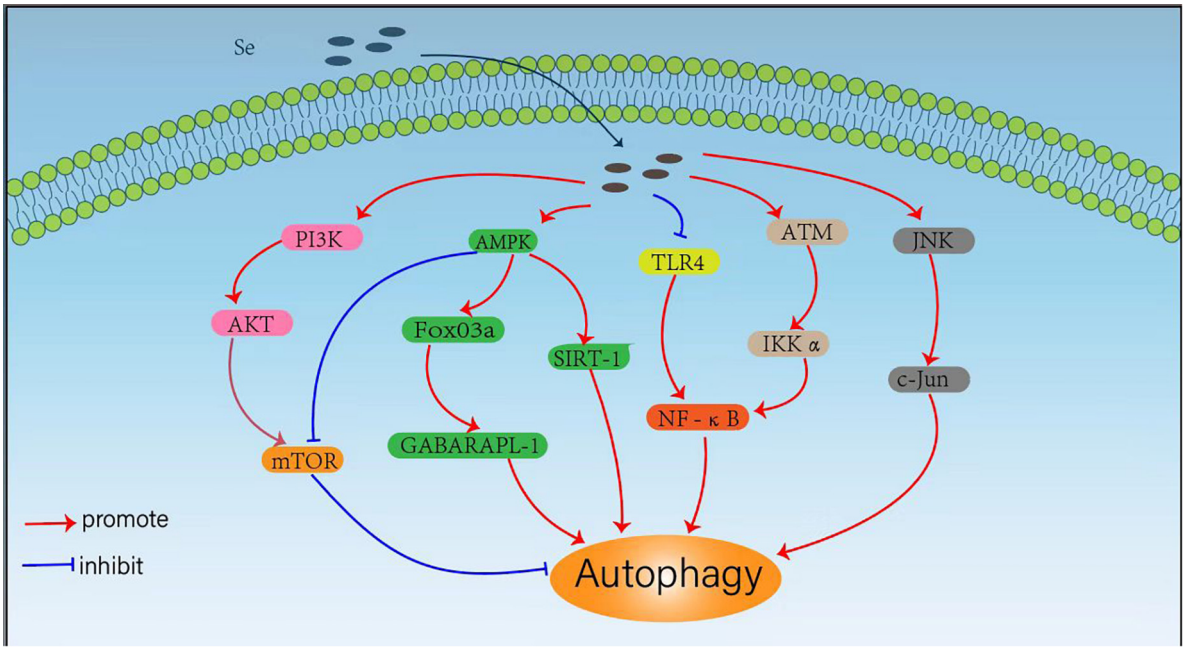

FIGURE 1
Mechanisms for selenium (Se) regulated cellular autophagy.

Mammalian rapamycin (mTOR) kinase is a well-known negative central regulator of the autophagic process, and its upstream regulatory signals are phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, which can activate mTOR signalings. The inhibition of PI3K/AKT/mTOR pathway activates autophagy, whereas AMPK downregulates mTOR expression and promotes autophagy by inhibiting the mTOR pathway (41). Qian et al. (22) observed that SeMet could attenuate OTA-induced PCV2 replication by inhibiting autophagy through activation of the PI3K/AKT/mTOR pathway. Cheng et al. (23) also found that L-selenomethionine could inhibit ammonia-induced cardiac autophagy through activation of the PI3K/AKT/mTOR signaling pathway. Yang et al. (42) also found that selenium could inhibit autophagy by regulating the PI3K/AKT/mTOR signaling pathway to prevent ischemia/reperfusion injury-induced blood-brain barrier damage in hyperglycemic patients. It has also been shown that SeMet can upregulate AMPK expression and initiate autophagy to clear tau in 3xTg-AD mice by activating the AMPK/mTOR pathway, suggesting that SeMet might be an effective drug candidate for the treatment of Alzheimer's disease (AD) (43). Se-Allylselenocysteine (ASC) can also activate autophagy and induce rectal cancer cell death by regulating AMPK/mTOR pathway, which facilitates the development of new therapeutic strategies against colorectal cancer (44).

Another downstream signaling pathway of AMPK is FoxO3a/GABARAPL1, and the activation of AMPK/FoxO3a/GABARAPL1 pathway can promote cellular autophagy. Yu et al. (26) found that sodium selenite could promote ROS/AMPK/FoxO3a/GABARAPL-1-mediated autophagy to downregulate apoptosis in colon cancer cells and colon xenograft models. In addition, Pant et al. (45) also found that probiotics enriched with selenium could improve hepatic steatosis by regulating AMPK/SIRT-1 mediated autophagy to alleviate NAFLD. The transcription factor NF-κB (nuclear factor kappa B) is not only a regulator of inflammatory response, but also plays an important role in the regulation of autophagy (46). It has been shown that selenium deficiency can induce chicken French bursa autophagy through the ChTLR4/MyD88/NF-κB pathway (47), and sodium selenite can also block TLR4/NF-κB and mitochondrial signaling pathways to inhibit mitochondrial autophagy in bovine support cells exposed to microcystins leucine arginine (MC-LR) (48). Selenite can also regulate the NF-κB signaling pathway by activating the ATM/IKK alpha axis to promote autophagy in leukemic Jurkat cells (49). Moreover, selenium donors can also induce macrophage autophagy through c-Jun-mediated pathway, thereby upregulating c-Jun expression and limiting the intracellular growth of Mycobacterium tuberculosis (25).

Autophagy is an important physiological process that delivers cytoplasmic or invasive materials into autolysosomes for degradation, which is critical to maintain the normal metabolism and thus beneficial to human health. However, in some pathological conditions, abnormal regulations of autophagy may also be harmful to human health. Se has exhibited powerful protective effects on the organism through its dual-role regulation effects on autophagy. It has been shown that maternal Se supplementation during gestation increases the levels of antioxidant selenoproteins and reduces autophagy and inflammation levels in the offspring, which helps to improve the immune function of the offspring (50), whereas maternal Se deficiency induces the dysfunction of autophagy and the damages of placenta (51). Se can also mitigate the damage caused by toxic substances such as cadmium (52–54), lead (55, 56), mercury (57), and ammonia (23, 58) through the inhibition of these toxins-induced and oxidative stress-mediated autophagy. In addition, Se may also attenuate oxidative stress, and ochratoxin A-induced PCV2 replication promotion by inhibiting autophagy (22, 59), which may be beneficial in developing new ideas for the prevention of PCV2 infection. These results indicated that Se can block unhealthy autophagic pathways to protect human health. It has also been shown that Se can exert antitumor effects by promoting autophagy

in different tumor cells for more effective tumor inhibition. Sodium selenite can induce autophagy not only in A549 human lung cancer cells by promoting ROS production (60), but also in colorectal cancer cells (26, 61), and these findings could help explore sodium selenite as a potential anti-cancer agent in clinical practice. In addition, Se not only limits the proliferation of Staphylococcus aureus and Mycobacterium tuberculosis by promoting macrophage autophagy (24, 25) but also alleviates the *E. coli*-induced inflammatory response by enhancing bovine mammary epithelial cell autophagy (62), which exhibits potent anti-infective activity. These results suggest that Se can not only block unhealthy autophagic pathways to protect human health, but can also promote healthy autophagic pathways to protect human health.

Mechanisms for Se NPs regulated autophagy in tumor

Similar with the conventional inorganic or organic selenium compounds, Se NPs can also modulate cellular autophagy through various signaling pathways. Despite being a promising strategy to inhibit cancer progression, current chemotherapy strategies for cancer are still limited due to adverse side effects and poor survival rates. Thus, the development of novel strategies, such as drug-delivery platforms with good biocompatibility that could enhance chemotherapy efficiency and reduce the side effects against cancer, remain a challenge. Wang et al. (63) developed a novel selenium-nanoparticle based drug-delivery agent for cancer treatment from *Kaempferia parviflora* (black ginger) root extract and selenium salts. The obtained KP-Se NPs showed significant cytotoxicity in human gastric adenocarcinoma cells (AGS cells), but showed no significant cytotoxicity in normal cells. These specific anticancer effects of KP-Se NPs were closely related to apoptosis, which was associated with the upregulation of intrinsic apoptotic signaling markers, such as Bcl-2, Bax and caspase 3 in AGS cells. Moreover, KP-Se NPs also caused autophagy of AGS by increasing the autophagic flux-marker protein, LC3B-II, whilst inhibiting autophagic cargo protein, p62. Additionally, phosphorylation of PI3K/Akt/mTOR pathway markers and downstream targets were decreased in KP-SeNP-treated AGS cells. Therefore, this work strongly suggested that KP-Se NPs could act as a novel potential therapeutic agent for GC by regulating autophagy, and also indicated that plant-based synthesis of Se NPs could be considered as one of the best strategies for cancer treatment.

Silymarin is well-known as a traditional hepatoprotective agent, but increasing evidence and clinical results have established the chemopreventive roles of silymarin as a therapeutic option for gastric cancer treatment (64, 65). However, silymarin has major limitations in clinical cancer therapy due to its poor water and lipid solubility (66). Therefore, Mi et al. (67) developed silymarin selenium nanoparticles (Si-Se NPs) by silymarin-mediated green synthesis and investigated their possibility as an anticancer agent. Compared with silymarin, the Si-Se NPs exhibited significantly increased cytotoxic effect of AGS cells without exhibiting toxicity on normal cells. Here, Se NPs could act as a carrier of silymarin to enhance the biocompatibility of silymarin, and silymarin could also further increase the anticancer effects of Se NPs. These Si-Se NPs were also proved to induce autophagy in AGS cells through the inhibition of PI3K/Akt/mTOR pathway. Their results demonstrated that Si-Se NPs could induce stronger inhibition effects on the phosphorylation of PI3Kand Akt than that of silymarin in gastric adenocarcinoma cells, which indicated that the loading of silymarin onto Se NPs could further enhance the anticancer effects of silymarin by inhibiting PI3K/Akt/mTOR pathway. These results demonstrated that the anti-cancer activities of Se NPs are closely associated with their ability to promote cancer cell autophagy by inhibiting PI3K/Akt/mTOR pathway, which could be combined with some anticancer agents, such as silymarin, for more effective PI3K/Akt/mTOR pathway inhibition and autophagy induction in cancer cells.

It is worth noting that the reports of organic molecules composing diselenide-containing nanoparticles can also be applied as autophagy regulators. diselenide-containing fluorescent molecules (SeBDP) and antitumor drug paclitaxel (SePTX) were synthesized and used for constructing SeBDP nanoparticles (SeBDP NPs) and SePTX NPs in aqueous solution through nanoprecipitation method (68). The cellular proliferation inhibition toward tumor cells (including HeLa and MCF-7 cells) was obviously higher than that toward normal cells (BEAS-2B and L929 cells), which might be attributed to the increasing reactive oxygen species in cancer cells treated by Selenium containing nanoparticles. Moreover, they further found that SePTX NPs can successfully induce oxidative stress, cause mitochondrial dysfunction, resulting in mitochondrial pathway-mediated apoptosis, which is related to the upregulation of autophagy-related protein LC3-II (69). These data suggested that SePTX NPs exhibited high inhibiting efficiency against the growth of tumors and were able to reduce the side effects by enhancing cancer cell autophagy.

Se and curcumin have both showed excellent antitumor effects individually or in combination with other therapeutic agents. Based on these two aspects, Kumari et al. (70) synthesized curcumin-supported selenium nanoparticles (SeCurNPs) to achieve an enhanced therapeutic effect. They found that the therapeutic effect of SeCurNPs on colorectal cancer cells (HCT116) was mainly attributable to increased levels of autophagy and apoptosis as autophagy-associated protein (LC3B-II) and proapoptotic protein (Bax) were significantly upregulated and anti-apoptotic protein (Bcl-2) and cytochrome C (Cyt C) were downregulated (70). These results indicated the ability of SeCurNPs for tumor treatment by increasing cancer cells autophagy. In the following work, they further synthesized CD44-targeted DOX loaded nanoparticles (PSHA-DOXNPs) and evaluated their anticancer efficacy in combination with curcumin loaded selenium nanoparticles (Se-Cur NPs) (71). Combination of these nanoparticles (NPs) increased ROS level, decreased mitochondrial membrane potential, induced cell cycle arrest and apoptosis in HCT116 cells, and also enhanced the autophagy of these cancer cells. These results also demonstrated the potential of Se NPs to work with other nanoparticles for synergetic anticancer treatment by regulating autophagy.

Huang et al. (72) synthesized Pleurotus tuber-regium (PTR)-conjugated Se NPs (PTR-Se NPs) and investigated its application in colorectal cancer. They found that PTR-Se NPs can trigger intracellular G2/M phase arrest and promote HCT 116 cell death by activating autophagy through upregulation of intracellular Beclin 1. In addition, they also found that autophagy plays an important role in the apoptotic promotion and induction of cell death by PTR-Se NPs, which can promote the release of pro-apoptotic proteins Bax and Bak and initiate mitochondria-mediated apoptosis (72). This study confirmed the high efficacy of PTR-Se NPs in the treatment of colorectal cancer and found that autophagy plays an important role in promoting apoptosis to induce cancer cell death. In same

specific situation, Se NPs could both be autophagy activators and inhibitors. Cui et al. (39) synthesized laminarin polysaccharides (LP) decorated selenium nanoparticles (LP-Se NPs) and found that these nanoparticles could induce mitochondria-mediated apoptosis by upregulating Bax expression, cutting caspase-9, and downregulating Bcl-2 expression. More interestingly, LP-Se NPs can induce early autophagy activation but block late autophagy in these cancer cells. The inhibition of autophagy in the late stage could lead to the failure of damaged organelles to be cleared, which could further aggravate cell apoptosis. The results suggest that LP-Se NPs play a cytotoxic role by inhibiting autophagy and promoting apoptosis.

Biomimetic materials are often capable of subtly affecting tissue development, and regeneration, and are also expected to mediate tumor suppression due to their high similarity to natural tissues. Li et al. (73) prepared hierarchically constructed bone-mimetic selenium-doped hydroxyapatite nanoparticles (B-SeHANs), which recapitulated the uniaxially oriented hierarchical structure of bone HA and could potentially play a dual role in the postoperative treatment of bone tumors *via* the chemotherapy from selenium and the promotion of bone repair by hydroxyapatite. In this work, they found that B-SeHANs could induce excess ROS production and promote autophagy in human MNNG/HOS osteosarcoma cells by activating ROS-mediated JNK pathway and inhibiting Akt/mTOR pathway. This work introduces a viable strategy for the development, evaluation and fundamental study of biomimetic selenium nanoparticles to inhibit tumor growth. Huang et al. (74) proposed dual-targeted modified selenium nanoparticles (u/A-Se NPs) as a biocompatible tumor chemotherapeutic drug, which was proved to promote ROS accumulation and autophagosome formation for synergistic HepG2 cell death. These results collectively suggested that Se NPs might be served as a novel anti-tumor agent due to their ability to promote cancer cell autophagy, which is closely related to their ability to induce high ROS levels.

Selenite, a touted cancer chemopreventive agent, has multiple mechanisms of cytotoxicity in cancer cells that are thought to be induced by selenite metabolites. Bao et al. (75) found that the intracellular metabolism of selenite can produce endogenous Se NPs in cancer cells, and its chelation with heat shock protein 90 can reduce the expression of LC3-II, showing the ability of these endogenous Se NPs for the inhibition of autophagy. In addition, Se NPs can also induce glycolytic inhibition and glycolytic-dependent mitochondrial dysfunction, suggesting that endogenous Se NPs may be the main cause of selenite-induced cytotoxicity. Although the exact mechanisms for how these endogenous Se NPs inhibit cancer cell autophagy remain to be further investigated, this work also introduces the potential for cancer cell inhibition by manipulating the endogenous Se NPs sysnthesis.

We have summarized the current knowledge of how Se NPs inhibit cancer cell growth or induce cancer cell death by promoting cancer cell autophagy (**Figure 2**). Se NPs are capable of activating ROS-mediated JNK pathway and inhibiting PI3K/Akt/mTOR pathway. These autophagy induction properties of Se NPs could further promote cancer cell apoptosis, which finally results in cancer cell death. Moreover, the ability of Se NPs to promote cancer cell autophagy by activating ROS-mediated JNK pathway and inhibiting PI3K/Akt/mTOR pathway might also be combined with current chemotherapy method for synergetic anticancer treatment.

Mechanisms for Se NPs regulated autophagy in infectious diseases

Except for the ability of Se NPs to inhibit cancer cells by regulating autophagy, our previous work also indicated the potentials of Se NPs to inhibit intracellular pathogens. Using some immune escaping mechanisms, such as their ability to inhibit autophagy and apoptosis of the host cells, Mycobacterium tuberculosis (Mtb) can escape from the immunological killings of host cells. We previously prepared a kind of Se NPs modified with mannose and encapsulated with isoniazide (Ison@Man-Se NPs) for synergistic drug-induced and phagolysosomal destruction of Mtb. Except for the ability of Ison@Man-Se NPs to promote Mtb-lysosome fusion, apoptosis and M1 polarization in Mtb infected macrophages, we also found that Ison@Man-Se NPs could promote autophagy of Mtb infected macrophages (76). The obtained results also indicated that Se NPs promoted autophagy might be associated with PI3K/Akt/mTOR pathway. And the formation of autophagosomes in the Mtb infected macrophages could finally promote the destruction of Mtb for synergistic intracellular Mtb killings with the anti-tuberculosis drugs. These results indicated that Se NPs, with the ability to promote autophagy of Mtb infected macrophages, could also serve as a kind of novel anti-tuberculosis agents for anti-tuberculosis strategy development. Moreover, these results also indicate the ability of Se NPs for intracellular pathogen clearance by regulating autophagy, which therefore can be served as potential anti-infectious agents.

Mechanisms for Se NPs regulated autophagy in other diseases

Rheumatoid arthritis (RA), a chronic autoimmune disease, is still lack of effective treatments. Liu et al. (77) prepared Se NPs-PEG-RGD@Ru by modifying selenium nanoparticles with PEG, RGD, and Ru, to target the abundant neovascular network of inflammatory sites, which increased NO production to activate autophagy by modulating signaling AMPK and mTOR pathways and inhibiting the activity of NF-κB-p65 to modulate the levels of inflammatory cytokines in Human Umbilical Vein Endothelial Cells (HUVECs). These unexpected results provided an effective strategy for the target treatment of RA based on the ability of Se NPs to promote autophagy of HUVECs.

Adolescence, a period of intense development, is accompanied by important physiological endocrine, and neurodevelopmental changes. Obesity, insulin resistance, and anorexia nervosa are particularly rising during this period. Ojeda et al. (78) firstly demonstrated *in vivo* that low selenite supplementation could promote adipogenesis through the insulin signaling pathway and lipocalin2 regulation, while low Se NPs administration could prevent fat storage in white adipose tissue and alleviate inflammation by reducing insulin signaling pathway and FOXO3A autophagy. These results provided data for dietary approaches to prevent obesity and/or anorexia during adolescence using Se NPs as autophagy regulators and also highlighted the potential roles of Se NPs for the prevention of adipocyte differentiation.

Se NPs may have a potential role in treating dermal disorders due to their wide therapeutic properties, but there is a need to evaluate their toxicity in keratinocytes. Thus, Kirwale et al. (79) synthesized Se NPs and tested their effects on keratinocytes, which indicated that Se

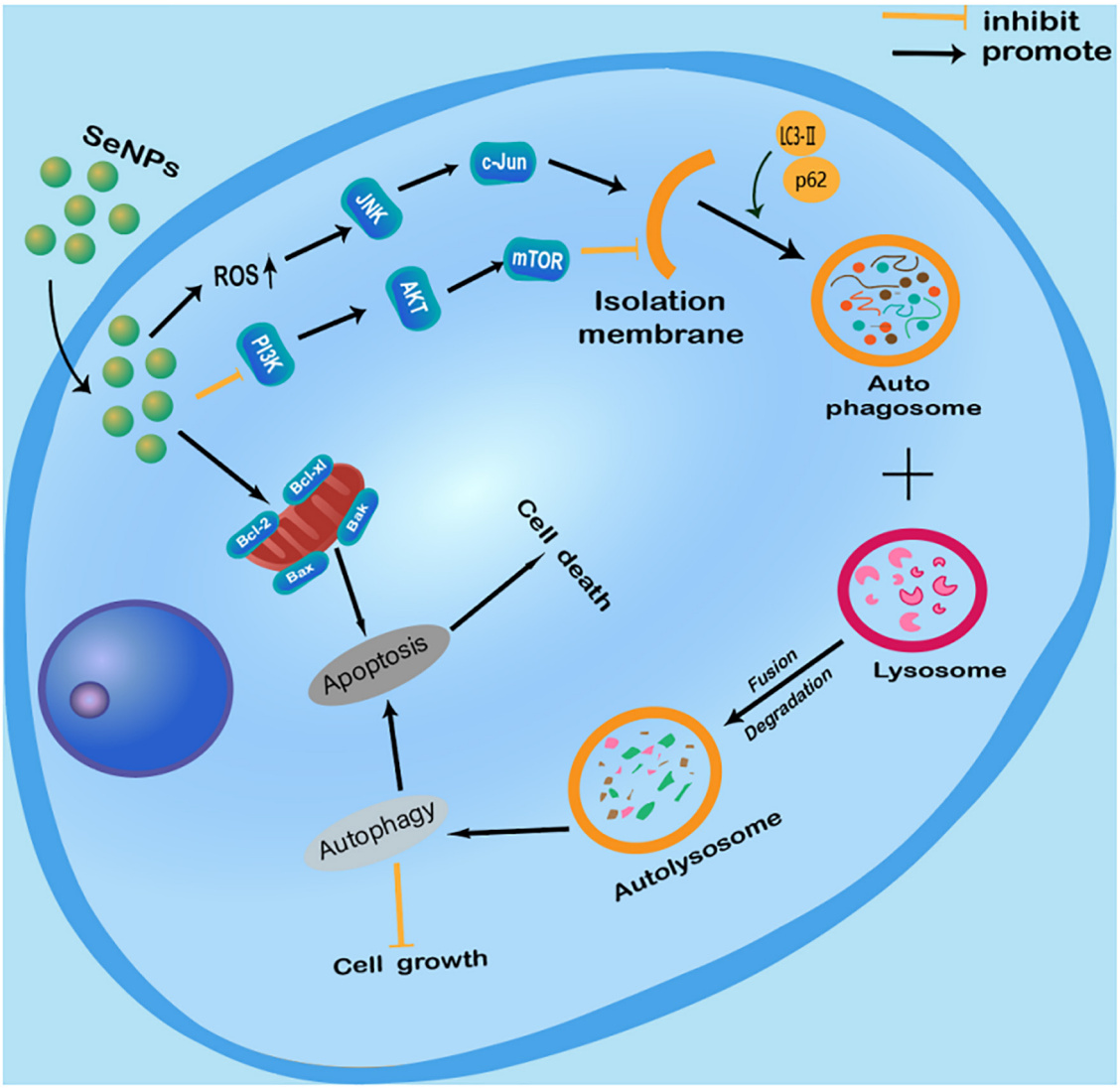

FIGURE 2
Anticancer effects of selenium nanoparticles (Se NPs) by promoting cancer cell autophagy through the regulation of reactive oxygen species (ROS)-mediated c-Jun N-terminal kinase pathway and PI3K/Akt/mTOR pathway.

NPs could promote autophagy by inducing AMPK phosphorylation and acidic lysosome formation, which finally enhanced the apoptosis of keratinocytes. These results indicated that Se NPs could induce the oxidative stress and autophagy mediated apoptotic cell death in human keratinocytes cells, which reminds the toxicity issues of Se NPs for further therapeutic application against dermal disorders.

Moreover, Se NPs can also show different protection effects by regulating autophagy. Yan et al. (80) found that Se NPs synthesized by Lactobacillus casei ATCC 393 could attenuate the H_2O_2-induced intestinal epithelial barrier dysfunction and ROS overproduction, as well as alleviate the adenosine triphosphate (ATP) level and the mitochondrial membrane potential (MMP) decrease. In addition, these Se NPs inhibited H_2O_2-induced phosphorylation of the mammalian target of rapamycin (m-TOR), and the increased expression levels of UNC-51-like kinase 1 (ULK1), light chain 3 (LC3)-II/LC3-I, PTEN-induced kinase 1 (PINK1), and Parkin proteins (80). However, the reasons for the simultaneous increased expression of m-TOR and LC3-II are not well-investigated. It has been widely known that m-TOR can also promote necrosis (81), so we speculate that H_2O_2 may induce autophagy and cell necrosis simultaneously, and some unknown signaling events involved in necrosis may promote the expression of m-TOR. In conclusion, these results suggested that these Se NPs can effectively alleviate H_2O_2-induced intestinal epithelial barrier dysfunction by regulating mTOR/PINK1-mediated autophagy (80). The same group also found that Se NPs can inhibit protein kinase R-like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor 2 (eIF2α), and the expression levels of CHOP and p-PERK activating transcription factor 4 (ATF4) to inhibit endoplasmic reticulum stress (ERS) in intestinal epithelial cells exposed to H_2O_2 (82). And Se NPs were found to regulate endoplasmic reticulum stress-mediated mitophagy by inhibiting the AMPK/mTOR/PINK1 signaling pathway, thereby reducing intestinal epithelial barrier damage (82).

These results suggested the protective effects of Se NPs regulated autophagy against H_2O_2-induced cell dysfunction by regulating mTOR/PINK1-mediated pathway, PERK/eIF2α/ATF4 and AMPK/mTOR/PINK1 signaling pathway (**Figure 3**). Although more works are still needed to further elucidate the exact mechanisms

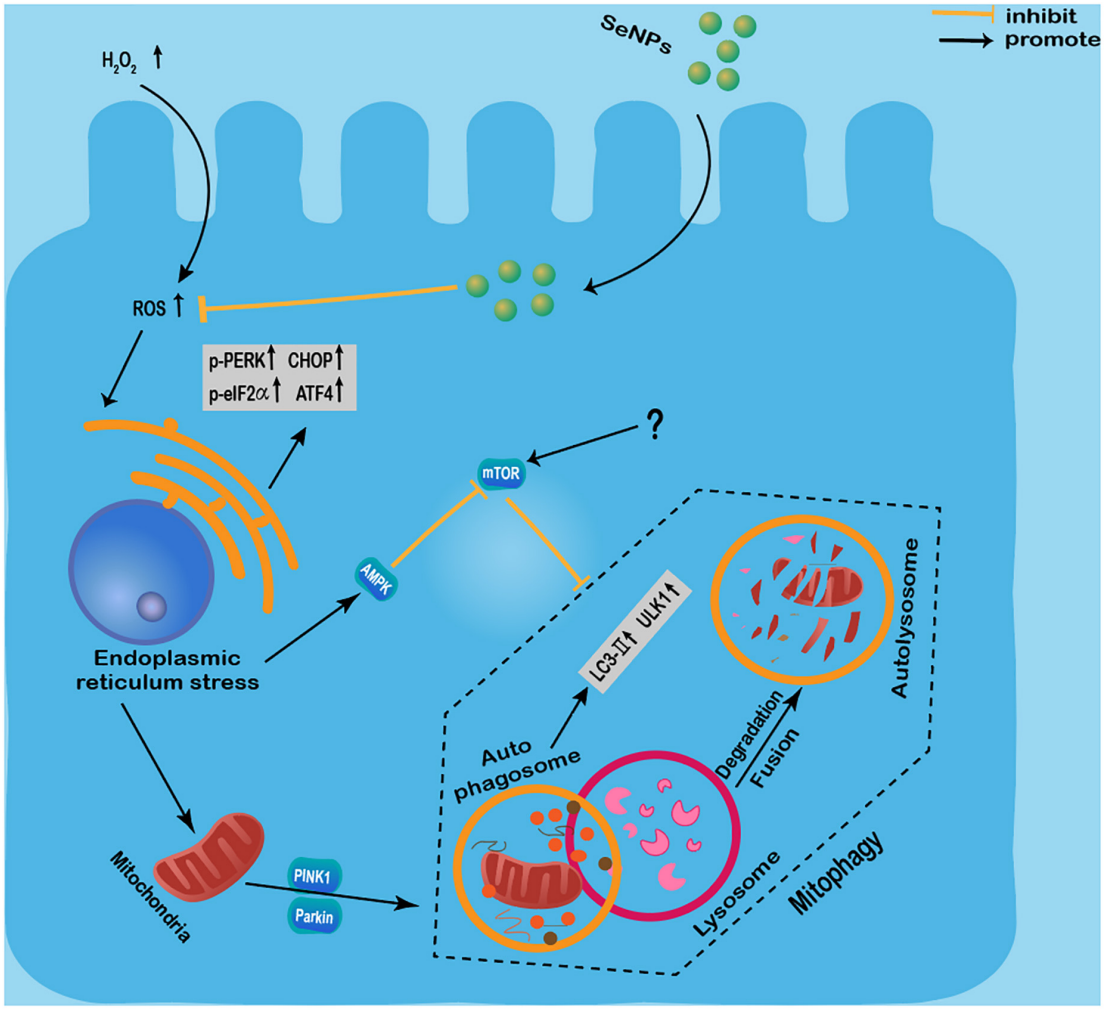

FIGURE 3
Protective effects of selenium nanoparticles (Se NPs) regulated autophagy against H_2O_2-induced cell dysfunction by regulating mTOR/PINK1-mediated pathway, PERK/eIF2α/ATF4 and AMPK/mTOR/PINK1 signaling pathway.

involved in their protective effects, the protective roles of Se NPs in different disease models by regulating autophagy have strongly suggested the potential uses of Se NPs as novel protective agents.

Potentials of Se NPs for disease treatment through autophagy modulation

Se NPs have attracted widespread attentions in biomedical field due to their unique biological, physical and chemical properties. By the modulation of autophagy, Se NPs exhibit excellent biological activities that can directly contribute to the treatment of cancer, infection and other diseases, which are expected to facilitate the development of novel therapeutics.

Cancer, one of the greatest threats to human health, is causing very high mortality rate worldwide that dramatically threatens human lives (83–85). Currently, the main treatment for cancer is chemotherapy, however, while chemotherapy kills cancer cells, it can also damage normal cells and cause serious side effects that also injure the human health (86–88). Se NPs, a potential anti-cancer agent, can not only directly kill cancer cells, but can also enhance the targeting effects of current chemotherapeutics and achieve synergetic anti-cancer therapy. For instance, adriamycin could be loaded onto Se NPs to achieve enhanced cellular uptake of adriamycin, which therefore resulted in greater anti-cervical cancer activity (89). By promoting the autophagy and apoptosis, Se NPs loaded with curcumin could kill the cancer cells more effectively and inhibit the tumor growth in tumor-bearing mice with prolonged survival time (70). In addition, the combination of curcumin loaded Se NPs and doxorubicin loaded Se NPs can target multiple molecular targets, which is beneficial to enhance anticancer effect (71).

Drug resistance of cancer is a widely-known issue that makes cancer cells to be tolerant to pharmaceutical treatment, which results in the more and more difficult therapy of cancer. Se NPs can also reduce the resistance of tumor cells to chemotherapy drugs by regulating autophagy. It has been shown that Se NPs modified with kelp polysaccharides can inhibit autophagy in HepG-2 cells by reducing the fusion of autophagosomes with lysosomes or decreasing the enzymatic activity of lysosomes, which is beneficial in reducing the resistance of tumor cells to chemotherapeutic agents *in vitro* (39).

Se NPs can also be combined with radiation therapy to increase the autophagy of breast cancer cells as a kind of novel radiosensitizer.

For example, Chen et al. (90) reported that Se NPs could reinforce the toxic effects of irradiation, lead to a higher mortality rate than either treatment used alone, induce cell cycle arrest and the activation of autophagy, and increase both endogenous and irradiation-induced reactive oxygen species formation for enhanced radiation therapy efficiency. PEG-modified Se NPs can synergistically inhibit tumor cell growth by DNA fragmentation and caspase-3 activation, which similarly shows radiosensitization to X-rays (91).

Infectious diseases, caused by pathogens infections such as bacteria, fungi and viruses, remain a serious diseases that threaten public health (92, 93). Plenty of works have indicated the ability of selenium compounds to kill intracellular pathogens by regulating the autophagy of pathogen infected host cells (22, 24). The antibacterial and antiviral activities of selenium compounds through modulation of autophagy indicate the potential ability of Se NPs for anti-infection treatments. It has been shown that macrophage targeted Man-Se NPs can kill Mycobacterium tuberculosis (Mtb) by promoting macrophage autophagy (76). This well-designed Se NPs can increase the expression of LC3B-II protein in BCG-infected THP-1 cells by regulating of PI3K/mTOR/AKT pathway, inducing autophagosome formation and promoting the fusion of Mtb into autolysosomes for more effective Mtb killing (76).

Diabetes is a chronic (long-lasting) health condition that affects how your body turns food into energy (94). It has been shown that Se NPs can alleviate the symptoms of weight loss, lower blood glucose levels and improve antioxidant status in diabetic mice (95). Diabetic nephropathy during pregnancy in diabetic female rats can also be alleviated by supplementation with Se NPs (96). These results suggest the promising application of Se NPs for anti-diabetic therapy. Due to critical roles of autophagy in diabetes development (97), we believe that more attentions would be paid into the effects of Se NPs on autophagy regulation for diabetes treatment.

Renal injury is a non-negligible issue for chemotherapy as lots of chemotherapeutic drugs would lead to inevitable renal toxicity (98). The intriguing relationships between Se physiology and several derangements and comorbidities associated with acute and chronic kidney disease have been well-demonstrated in recent years (99). Se NPs have also been proved to efficiently reduce renal tissue injury and regulate the expression pattern of aldose reductase in the diabetic-nephropathy rat model (100), indicating the potentials of Se NPs to relieve renal injury. In addition, Se NPs can also improve gentamicin-induced kidney damage by inhibiting oxidative damage, inflammation and apoptosis-induced by autophagy (101), which introduced a good option of Se NPs as an adjunctive treatment to reduce its side effects for kidney damage.

Conclusion and perspectives

Autophagy is an evolutionarily conserved process that mediates the degradation of long-lived proteins and damaged organelles in response to a variety of stressful stimulus, including starvation, oxidative stress, and viral infection (1, 102, 103). During autophagy, the cytoplasmic fraction is isolated in autophagosomes, which eventually fuse with lysosomal compartments for overall degradation (104, 105). Se is an essential trace element in animals, and has been proved to show important biological function in anti-oxidative stress, anti-tumor and improving the immunity of the body. Different forms Se (including sodium selenite, selenoprotein, and selenium-rich yeast) act in different ways in the human body, and Se NPs have attracted increasing of attention in recent years due to their attractive biological activities (26, 106, 107). Here, we summarized the current research progress on the ability and how could Se NPs induce tumor cell death, reduce drug toxicity, modulate inflammatory responses, resist pathogenic bacterial infections, treat Alzheimer's disease as well as other diseased conditions by regulating autophagy.

Although Se has showed attractive biological activities, selenium compounds are widely restricted by their high toxicity and poor targeting. Selenium compounds, such as sodium selenite, have strong anticancer effects only at high doses, but the toxicity at high doses just provided a weak gap between beneficial and toxic effects. Therefore, toxicity has been one of the key issues in the development of selenium-based anticancer drugs. Se NPs, a new type of monomeric selenium, have indicated strong biological activities, such as low toxicity, high bioavailability, regulation of selenoprotein functions, scavenging of free radicals, protection of oxidative DNA damage, and strong anti-cancer effects compared to inorganic or organic selenium compound (29, 108–110). In addition, taking the advantages of good biocompatibility, high loading rate, low toxicity, easy synthesis and easy storage, Se NPs can also serve as drug carriers to improve the solubility and stability with prolonged cycle time, thus increasing drug efficiency (33, 111, 112).

More importantly, increasing studies are suggesting that Se NPs can serve as a kind of novel autophagy regulators, which might provide the potential use of Se NPs against different diseased conditions. In recent years, the ability of Se NPs for autophagy regulation has been proved to show strong potentials in some impotent diseases, including cancer, tuberculosis, rheumatoid arthritis, adolescent obesity or anorexia, skin diseases, diabetes and kidney injury. The attractive bioactivity of Se NPs for autophagy modulation not only suggests Se NPs for disease treatment alone (78, 79), but also introduces the possibilities to combine Se NPs and current chemotherapeutics to achieve enhanced efficiency (70, 71), which would be beneficial in facilitating the development of new therapies.

However, there are still lots of critical issues for the further clinical application of Se NPs by regulating autophagy. Firstly, although some works have indicated the potential mechanisms of Se NPs induced autophagy, most of these works just provided some phenotype results without depth investigations (68, 75, 78). To further confirm the detailed mechanisms of Se NPs induced autophagy, knockout/knockdown experiments in cell and mice models are needed. These works would further explain whether these potential pathways are critical for the control of Se NPs induced autophagy. Secondly, the relations between autophagy and apoptosis are also needed to be further investigated in Se NPs induced autophagy, as most of the results indicated that Se NPs could both induce autophagy and apoptosis (63, 67, 73).

Thirdly, the toxicity issue of Se NPs should be considered as one of the most important issues for the further application of Se NPs. Although some works have indicated that Se NPs could inhibit cancer cells selectively without significant inhibition effects on normal cells (63, 89), the detailed mechanisms for these selectivity are still unknown. And there are still few studies that specifically focus on the systemic toxicity of long-term Se NPs treatment *in vivo*. Although Se NPs have been claimed to show relatively lower toxicity and higher degradability, the degradation and metabolism processes of Se NPs *in vivo* have

not been well-explored. More concerns should be paid to the safety issues of Se NPs to investigate how they uptake, metabolize, degrade and eliminate in animal models, as well as how they interact with normal tissues and cells *in vivo* and *in vitro*, on account of excessive Se causing toxic symptoms.

Overall, taking the advantages of their advanced ability to regulate autophagy, Se NPs have demonstrated attractive potentials for the treatment of different diseases. And these autophagy regulation effects of Se NPs can be served as an adjuvant treatment to boost the efficiency of current therapeutics, such as chemotherapy, radiotherapy and immunotherapy. With the increased understanding of their functions and mechanisms, especially their metabolism, degradation and long-term safety *in vivo*, we believe that Se NPs could be applied as kind of novel autophagy regulators, which might provide new possibilities to benefit the clinical therapeutics against some important diseases.

Author contributions

DC and HL drafted this manuscript. YM, SF, YuH, TZ, WL, and YiH helped to revise the manuscript. YR, J-FX, and JP helped to revise the manuscript and were responsible for leading this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Selenium-enriched plant foods: Selenium accumulation, speciation, and health functionality

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Selenium (Se) is an essential element for maintaining human health. The biological effects and toxicity of Se compounds in humans are related to their chemical forms and consumption doses. In general, organic Se species, including selenoamino acids such as selenomethionine (SeMet), selenocystine ($SeCys_2$), and Se-methylselenocysteine (MSC), could provide greater bioactivities with less toxicity compared to those inorganics including selenite (Se IV) and selenate (Se VI). Plants are vital sources of organic Se because they can accumulate inorganic Se or metabolites and store them as organic Se forms. Therefore, Se-enriched plants could be applied as human food to reduce deficiency problems and deliver health benefits. This review describes the recent studies on the enrichment of Se-containing plants in particular Se accumulation and speciation, their functional properties related to human health, and future perspectives for developing Se-enriched foods. Generally, Se's concentration and chemical forms in plants are determined by the accumulation ability of plant species. Brassica family and cereal grains have excessive accumulation capacity and store major organic Se compounds in their cells compared to other plants. The biological properties of Se-enriched plants, including antioxidant, anti-diabetes, and anticancer activities, have significantly presented in both *in vitro* cell culture models and *in vivo* animal assays. Comparatively, fewer human clinical trials are available. Scientific investigations on the functional health properties of Se-enriched edible plants in humans are essential to achieve in-depth information supporting the value of Se-enriched food to humans.

KEYWORDS

selenium, bioactivity, selenium-enriched, functional food, antioxidant, plant food, essential trace mineral, health

Introduction

Selenium is an essential trace element for human health. According to the World Health Organization (1), a recommended consumption level of Se is 55-70 μg day^{-1} for adults, with 400 μg day^{-1} as a toxic concentration. Selenium deficiency situation has transpired in some parts of the world, including China (about 72% of the area), Europe (e.g., France and Norway) and New Zealand (2). Selenium is associated with the normal function of glutathione protein (GSH) and its family of antioxidant enzymes such as glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and other selenoproteins (3). The lack of Se can severely affect the human immune system (4, 5), leading to a cardiomyopathy disorder called "Keshan disease" and the bone and joint connection syndrome called "Kashin-Beck disease" (6, 7). Keshan disease occurs when vascular endothelial cells are damaged from oxidative stress due to non-functional antioxidant proteins (8). This disease also causes some serious health problems such as atherosclerosis, hypertension, myocardial necrosis and congestive heart failure (9). Kashin-Beck disease is an endemic osteoarthropathy, causing severe symptoms to joints and bone, including joint pain, elbows flexion and extension disturbances, enlarged inter-phalangeal joints, and limited joint motion (10, 11). Moreover, Se deficiency also increases the risk of arthritis, cancers, and neurodegenerative disorders regarding immune and inflammatory infections (12, 13).

In contrast to Se deficiency, there are a few high soil Se regions globally. The prominent one being the Enshi Province in China, where the soil Se content can rise to 11.4 mg Se kg^{-1} in the high Se area (14). People live in the high Se soil area can suffer from selenosis symptoms and abnormal growth conditions due to excessive Se consumption of foods produced from the area (6, 15). The Se intake of Enshi people was reported to reach 833 μg per day (15), with serum Se concentrations of up to 41.6 μmol L^{-1}, approximately 20 times higher than the proposed intake (16). Chronic selenosis is a group of diseases associated with a wide range of symptoms from hair loss, bone and joint problems, and cellular damage from reactive oxygen species which increase the high risk of cancers (17, 18).

In general, toxicity associated with Se intake occurs in a few isolated areas, and food science and technology innovation can help lower Se imbalance intake in the diet. Selenium is present in plant foods in different chemical forms, including the organic Se-containing amino acids, i.e., selenomethionine (SeMet), Se-methylselenocysteine (MeSeCys), and γ-glutamyl-Se-methylselenocysteine (γ-GluMeSeCys), and the inorganic Se, i.e., selenite and selenate (19). Advanced analytical techniques are applied for identifying Se compounds in plant food samples nowadays, contributing to the knowledge of Se chemical forms present in plant foods, their content, and the safe concentration for human consumption. In developing Se-enriched food products, the aim should be focused on providing functional food products to benefit human health and enhance the quality of life. Identification of the Se chemical form and content is essential to justify the use of Se-enriched plant foods for achieving health benefits and overcoming the deficiency issues associated with this essential trace mineral. The objectives of this review are to examine the Se's accumulation ability and speciation in a wide range of Se-enriched plant foods, to inspect Se and Se compounds' biological effects on human health, and to explore the prospects of developing Se-enriched plant foods for health purposes.

Accumulation of selenium in plants

Over the past few decades, Se-enriched plants have been developed to demote deficiency problems for those living in low Se regions who cannot maintain the recommended intake level (18). One of the most simple and robust techniques to increase Se content in plants is by growing plants in high Se soil and applying Se fertilizers. This enrichment method relates to plant species' absorption, transformation, and accommodation ability of minerals (6). The Se accumulation ability of plants can be classified into three levels: hyper-accumulators, secondary accumulators and non-accumulators. The hyper-accumulators (e.g., *Stanleya, Astragalus*, *Conopsis, Neptunia*, *Xylorhiza*) can accumulate more than 1,000 mg Se kg^{-1} while the secondary accumulators (e.g., *Brassica juncea*, *Brassica napus*, *Broccoli*, *Helianthus*, *Aster*, *Camelina*, *Medicago sativa*) can accumulate between 100-1,000 mg Se kg^{-1}. The non-accumulators only accumulate less than 100 mg Se kg^{-1} and most of the angiosperm species are included in this category (20–22).

The metabolism of Se in plant species varies among plants, meaning that different plant varieties can produce different Se chemical forms in various concentrations. **Figure 1** demonstrates the complexity of Se chemical forms in different plant species. Literature on Se speciation revealed that the Brassica family, such as broccoli, cabbage, and radish, have MSC as the main Se compound stored in their cells, while SeMet is the main Se chemical form found in cereals grains and tuber crops such as ginger, wheat, and carrot (23–25). On the other hand, selenolanthionine is a major water-soluble Se compound found in *Cardamine violifolia* (26).

As the Se content and chemical form in plant materials are specific to the plant species and their metabolism pathways, we need to understand the Se accumulation mechanisms in the plant when selecting plant species for producing Se-enriched plant foods and food ingredients for human diets. The accumulation pathways of Se content start with the inorganic Se (i.e., selenite and selenate) in soil, which plants could uptake and transform into organic forms (i.e., selenocystine ($SeCys_2$), selenomethionine (SeMet), selenohomocysteine,

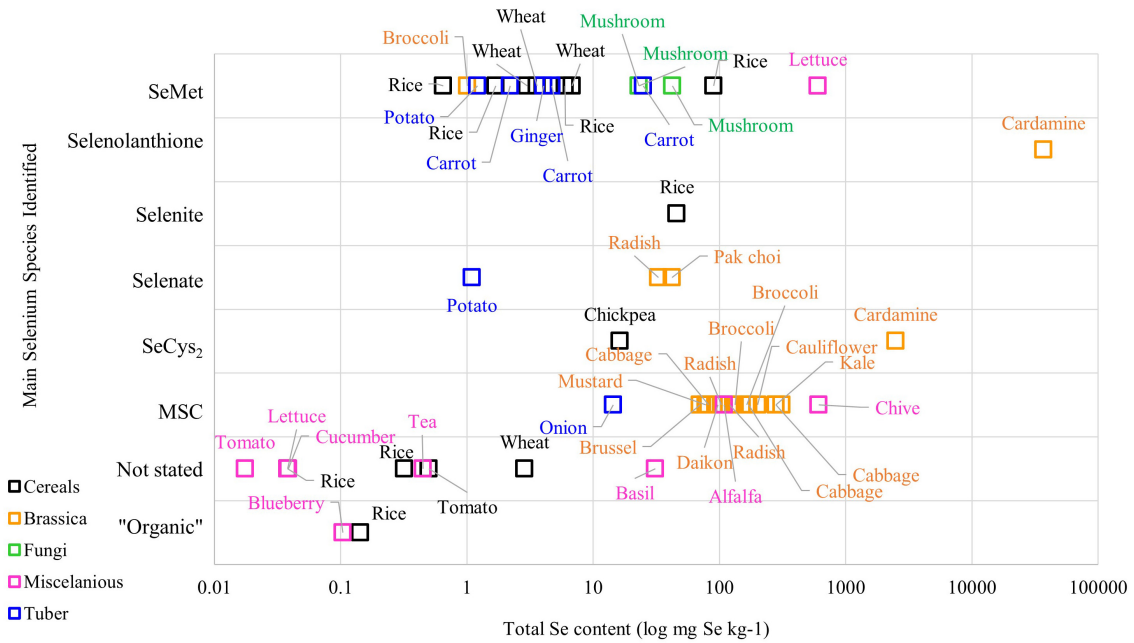

FIGURE 1
The Se content and chemical species in plant-based foods from the literature (Please refer to **Supplementary Table 1** for the original data from the literature).

selenolanthionine Se-methylselenocysteine (MSC) and γ-glutamyl-methylselenocysteine (γGMSC)) through the metabolic pathways as shown in **Figure 2**. Briefly, selenate and selenite are taken through the plant root via high-affinity sulphate transporter (HAST) and high-affinity phosphate transporter (PHT). Selenate is converted to adenosine 5'-phosphoselenate (APSe) via ATP sulfurylase (**Figure 2**, step 1), then changed to selenite through adenosine phosphosulfate reductase (**Figure 2**, step 2). Selenite is reduced to selenide (Se^{2-}) by sulphite reductase (**Figure 2**, step 3), and then it is transformed to selenocysteine (SeCys) by O-acetylserine thiol-lyase (**Figure 2**, step 4). SeCys could also be transformed to Se-cystathionine via cysthathionine-γ-synthase (**Figure 2**, step 5), MSC via selenocysteine-lyase (**Figure 2**, step 7), or elemental selenium (Se0). Secystathionine could then be changed into selenohomocysteine (SeHCys) via cysthathionine-β-lyase (**Figure 2**, step 6). MSC could be converted to dimethyldiselenide (DMDSe), a volatile compound and released from plant cells. SeCys is transported to the cytoplasm and is reacted with glutamic acid to form γ-glutamyl-Se-methylselenocysteine (γGMSC) by γ-glutamyl-cysteine synthetase (**Figure 2**, step 8). SeHCys can also be transported to the cytoplasm and synthesized to form selenomethionine (SeMet) by methionine synthase (**Figure 2**, step 9). SeMet could also be converted to methyl-selenomethionine (MSeMet) by methionine methyltransferase (**Figure 2**, step 10), then changed to the volatile dimethylselenoproprionate (DMSeP) and released as dimethyl-selenide (DMSe) via dimethylselenoproprionate-lyase (**Figure 2**, step 11) (27–29).

During the accumulation process, selenite tends to provide higher bioavailability than selenate, and it is commonly used as Se fertilizer for producing Se enriched plants (30, 31). Hu et al. (24) showed that using selenite as the foliar fertilizer on rice grain increased the Se concentrations in glutelin and albumin proteins as $SeCys_2$ and SeMet. Selenite could cause significant phytotoxicity from a generation of superoxide in plant cells during a non-enzymatic reduction reaction to produce selenide (25, 32, 33). In another study, Ramkissoon et al. (34) applied sodium selenate to wheat as foliar fertilizer and found an increased Se concentration and the highly bioavailable SeMet fraction in wheat grain. However, Se can cause cytotoxicity in plants and humans when accumulated or consumed excessively. At high concentrations, Se shows cytotoxicity by either generating reactive oxygen species or malformed selenoprotein (20). Generally, inorganic Se, either selenite or selenate, generates toxicity via the activation of ROS, which inhibits the growth rate and causes lipid oxidation related to malondialdehyde formation in plant tissue (35, 36).

In contrast, organic Se, such as SeMet and SeCys, cause toxicity to plant cells by forming malformed selenoproteins due to the replacement of Cys/Met with SeCys and SeMet in the peptide chain. Changing between Cys and SeCys changes cellular protein's structure by changing disulfide bond to diselenide bond to 60 mg Se kg^{-1}, which affects the peptide chain's redox potential. Moreover, SeCys is

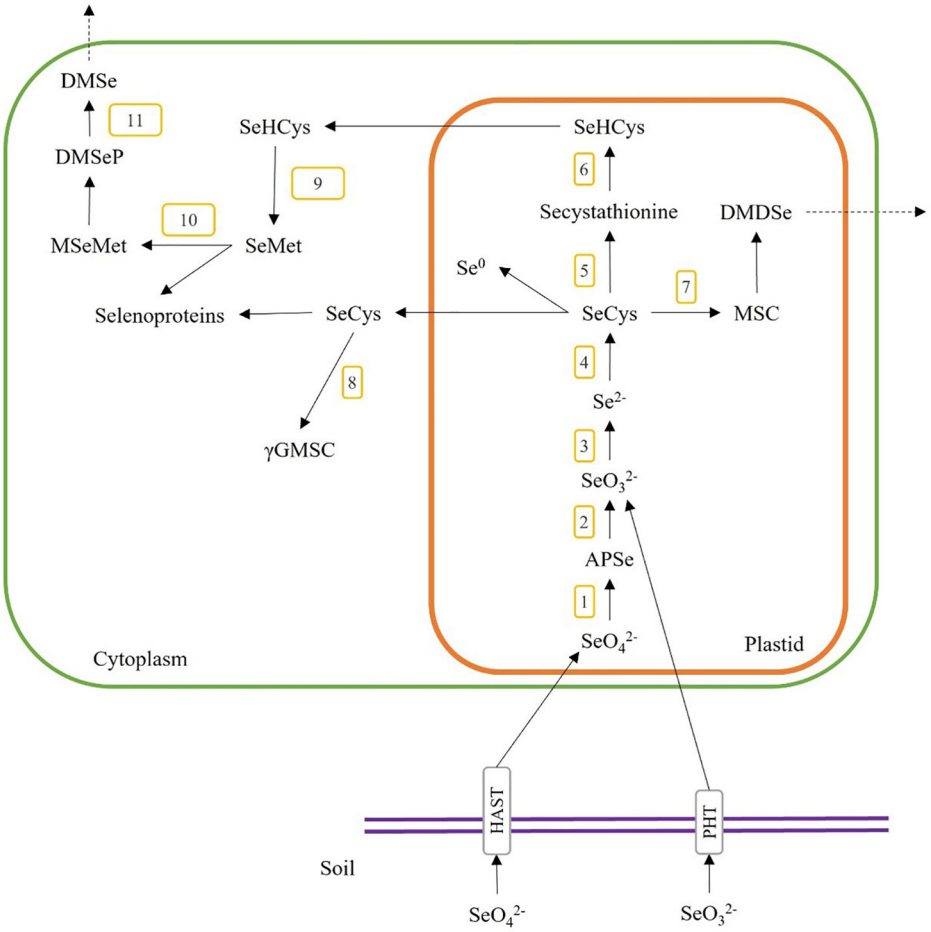

FIGURE 2
A general overview of Se uptake, metabolism, and incorporation in higher plants. The numbers 1–12 indicate the possible enzymatic steps involved in the conversion of selenite and selenate. 1, ATP sulfurylase; 2, adenosine phosphosulfate reductase; 3, sulfite reductase; 4, O-acetylserine thiol-lyase; 5, cystathionine-γ-synthase; 6, cystathionine-β-lyase; 7, selenocysteine-lyase; 8, γ-glutamyl-cysteine synthetase; 9, methionine synthase; 10, methionine methyltransferase; 11, dimethyl selenopropionate-lyase; SeO_4^{2-}, selenate; SeO_3^{2-}, selenite; APSe, adenosine 5′-phosphoselenate; SeCys, selenocysteine; MSC, Se-methylselenocysteine; DMDSe, dimethyldiselenide; SeHCys, selenohomocysteine; Se^0, elemental selenium; γGMSC, γ-glutamyl-methylselenocysteine; SeMet, selenomethionine; MSeMet, methyl-selenomethionine; DMSeP, dimethylselenopropionate; DMSe, dimethyl selenide.

more reactive than Cys, which could increase enzyme activity and the metal binding co-factor activity of malform selenoproteins (27). Literature has shown that organic Se's toxicity level is far less than inorganic ones because they can be capped with proteins and polysaccharides (37). Moreover, the organic Se compounds display a higher bioavailability than the inorganic Se (38). The organic Se involves in the upregulation of enzymatic antioxidant capacity which play a key role in Se tolerance (39). As the Se chemical forms significantly affect the biological activities of Se-enriched plants, it is essential to perform chemical speciation of Se compounds to gain scientific insight into the relationship between chemical forms and the functional properties of Se-enriched plant foods.

Speciation of selenium compounds

Speciation of Se compounds in Se-enriched plant foods has been studied to relate to and explain the biological activity of the products. Se can accumulate in plant organelle, stay either in free molecules form, or bound with a larger and more complex structure such as polypeptides or polysaccharides. Most inorganic Se compounds and small selenoamino acids such as selenolanthionine, γGMSC, MSC, $SeCys_2$ and SeMet are water-soluble molecules, therefore water extraction is a common method applied to separate these small molecules from the sources. Proven in some previous studies, extraction efficiencies in hot water ranged between 47 and 91% Se in different mushroom species (40); 40% for Se-enriched mycelium

TABLE 1 Cytotoxicity of Se compounds against human cancer cell lines.

Tumor organs	Cell lines	Se compounds	Effective doses (IC_{50})	Cell viability method	Mechanism of cell death	References
Lung	A549	SeMet	50 μM	MTT Assay	• Induce ROS generation • Induce ER stress-related to p53 regulation	(73)
		SeMet	50 μM	MTT Assay	• Induce ROS generation Interrupt PI3K/Akt/mTOR pathway	(117)
		SeMet	500 μM	MTT Assay	• Induce ROS generation	(74)
		SeMet	200 μM	Cell counting kit-8	• Induce ROS generation • Increase the intensity of the mitochondrial membrane	(118)
		MSC	50 μM	MTT Assay	• Activate caspase-3,-8,-9 • Interrupt PI3K/Akt pathway • Induce ER stress	(73)
		$SeCys_2$	5 μM	ATP measurement	• Induce ROS generation • Decrease total cellular glutathione	(119)
		$SeCys_2$	8 μM	MTT Assay	• Induce ROS generation • Induce loss of mitochondria membrane by regulating Bcl-2 family proteins • Induce apoptosis via inactivating ERK and AKT pathways	(77)
		MSA	2.2 μM	MTT Assay	• Induce DNA single strand break • Induce apoptosis via cell cycle arrest G1 phase	(120)
		Nano-Se	4 μM	MTT Assay	• Induce apoptosis via cell cycle arrest G2/M phase	(121)
	95-D	MSA	4 μM	MTT Assay	• Induce ROS generation and oxidative damages	(122)
Breast	MCF-7	$SeCys_2$	10 μM		• Induce H_2O_2 production • Decrease mitochondria protein UCP2 and MnSOD	(123)
		$SeCys_2$	16.2 μM	MTT Assay	• Induce DNA single strand break • Induce ROS generation • Decrease cellular antioxidant enzymes	(124)
		MSA	2 μM	FACS CANTO II	• Induce apoptosis via cell cycle arrest G2/M phase • Inhibit DNA methyltransferase 1 (DNMT1)	(125)
	MCF-7	SeMet	45 μM	SRB Assay		(126)
		$SeCys_2$	40.8 μM	CCK-8 assay	• Induce apoptosis via cell cycle arrest G1 phase	(127)
Colon	HCA-7	SeMet	60 μM	SRB Assay	• Inhibit cyclooxygenases-2 (COX-2) protein	(128)
	HT-29	SeMet	130 μM	SRB Assay		(126)
	HCT116	SeMet	100 μM	Propidium iodide staining	• Induce apoptosis via cell cycle arrest G2/M phase • Decrease mitotic cyclin B RNA expression • Decrease cdc2 kinase activity	(129)
	SW620	$SeCys_2$	7.3 μM	MTT Assay	• Induce DNA single-strand break • Induce ROS generation • Decrease cellular antioxidant enzymes	(124)
	Colo201	$SeCys_2$	27.8 μM	MTT Assay	• Induce DNA single-strand break • Induce ROS generation • Decrease cellular antioxidant enzymes	(124)
Prostate	LNCaP	SeMet	50 μM	Model Z F Coulter Counter	• Increase p53 gene expression	(130)
		SeMet	1 μM	Growth Inhibition Assay	• Induce apoptosis via cell cycle arrest G2/M phase	(131)
	DU145	SeMet	40 μM	SRB Assay	–	(126)
		SeMet	90 μM	Growth Inhibition Assay	• Induce apoptosis via cell cycle arrest G2/M phase	(131)
		MSA	5 μM	MTT Assay	• Induce apoptosis via inactivation of protein kinase C (PKC)	(132)
	PC-3	SeMet	70 μM	Growth Inhibition Assay	• Induce apoptosis via cell cycle arrest G2/M phase	(131)

(Continued)

TABLE 1 (Continued)

Tumor organs	Cell lines	Se compounds	Effective doses (IC_{50})	Cell viability method	Mechanism of cell death	References
Liver	HepG2	$SeCys_2$	17.5 μM	MTT Assay	• Induce DNA single-strand break • Induce ROS generation • Decrease cellular antioxidant enzymes	(124)
		Selenosulfate	>15 μM	MTTAssay	–	(133)
Bone	MG-63	$SeCys_2$	20 μM	MTT Assay	• Induce apoptosis via cell cycle arrest G2/M phase • Decrease cyclin A and CDK-2, PARP cleavage, and caspases activation	(134)
Urinary bladder	T24	Selenosulfate	3.5 μM	MTT Assay	–	(133)
Brain	IPSB-18	Sodium selenite	4 μg/ml	MTT/SRB Assay	• Downregulation metalloproteases genes and epidermal growth factor receptor	(135)
Oral	HSC-3	MSC	>50 μM	MTTAssay	• Enhance activity of caspase-3, -8, -9 • Induce ER stress • Reduce phosphorylated Akt levels and vascular endothelial growth factor (VEGF).	(73)
Skin	UACC-375	SeMet	50 μM	SRB Assay	–	(126)
	A375	$SeCys_2$	12.8 μM	MTT Assay	• Induce DNA single strand break • Activate caspase peptides • Induce p53 expression	(136)
		Selenosulfate	4.7 μM	MTT Assay	–	(133)
		$SeCys_2$	20 μM	MTT Assay	• Upregulate genes encoding cell death and transcription factors • Downregulate cell development, cell adhesion and cytoskeleton genes	(137)
Cervix	HeLa	$SeCys_2$	99.5 μM	XTT cell proliferation kit II	• Upregulate apoptosis gene BCL2L11 and DNA damage GADD45G • Induce cytoplasmic vacillation via LC-3II protein formation • Induce ER stress by decreasing ER-residing protein	(138)

SeMet, Selenomethionine; MSC, Se-methylselenocysteine; $SeCys_2$, selenocystine; MSA, methylseleninic acid; ROS, reactive oxygen species.

(*Lentinula edodes* (Berk.) *Pegl.*) (41), 85% for Se-enriched garlic (42) and 60% for *Cardamine violifolia* (26). Multiple sample preparation steps have been used to release Se bind to some larger components in plant cell walls. For example, hydrolysis of polysaccharides using an enzyme such as cellulase, hemicellulose, β-glucanase and pectinase, has been applied to hydrolyze plant cell walls, followed by protease enzymes to release selenoamino acids (43, 44).

Selenium compounds extracted from the plants could be separated by the High-Performance Liquid Chromatography (HPLC) technique, commonly used in the chemical compound analysis. Various types of chromatography resin can be used to separate the specific Se compounds in plants. For example, ion-exchange chromatography is used in the scouting period, which can classify Se chemical compounds according to their electron charge binding to ion exchange resins, either in a negatively charged resin (cation exchanger) or a positively charged resin (anion exchanger) (45, 46). Thus, ion-exchange chromatography is the technique that separates Se molecules by the positively or negatively charged groups retained on a stationary phase in equilibrium with free counter ions in the mobile phase (47). Generally, when the pH of the eluent buffer is higher than the pKa of the molecule, the compound shows a negative charge and binds to the positive charge anion exchanger (46, 48). Anion exchange liquid chromatography has a positively charged stationary phase to interact with the negatively charged Se compounds, such as selenate ($pKa = 1.92$), selenite ($pKa = 2.46$) or SeMet ($pKa_1 = 2.19$ and $pKa_2 = 9.05$) in the deprotonated state which can be strongly retained on anion exchange resin at pH around 5. In contrast, Se compounds with higher pKa values, such as $SeCys_2$ ($pKa \sim 8.07$ and 8.94), will be in protonating state and retained very little on anion column chromatography at pH around 5 in the mobile phase (49, 50). In contrast, cation exchange chromatography works similarly to anion exchange, except that the stationary phase is negatively charged, which could interact with the positively charged Se compounds (51, 52). Furthermore, some other types of chromatography could be applied for Se compound separation. For example, size exclusion chromatography is used to separate compounds based on their particle size; reversed phase and hydrophilic interaction chromatography could be applied to separate Se compounds based on the polarity of their molecules (51). These types of chromatography can be

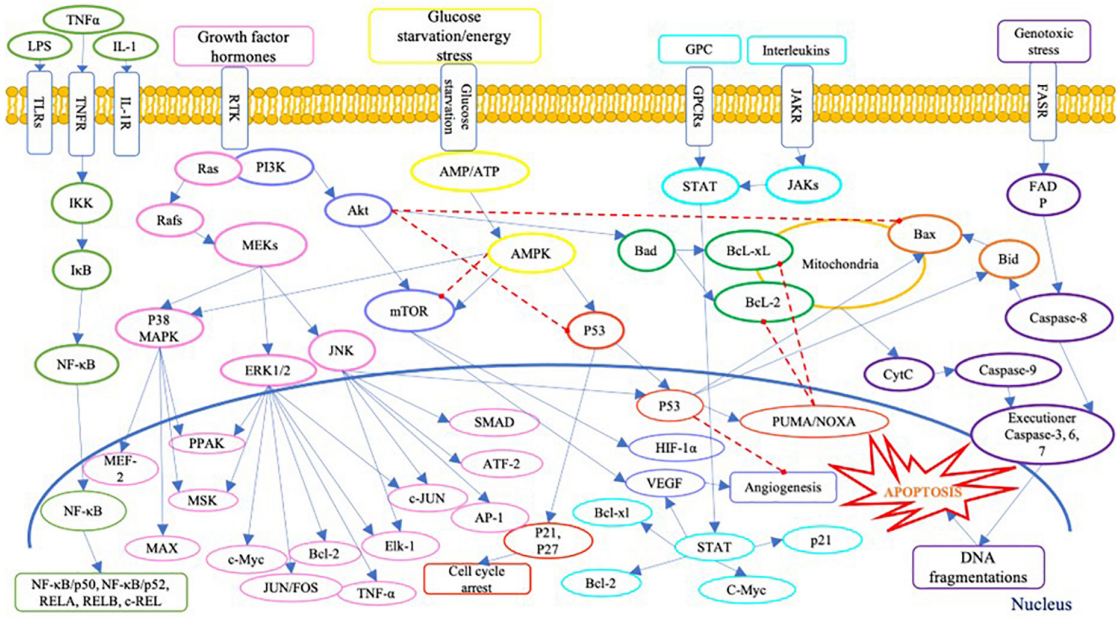

FIGURE 3
A schematic of apoptosis signaling pathways. LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha; IL-1, interleukin-1; TLRs, Toll-like receptors; TNFR, tumor necrosis factor receptors; IL-1R, interleukin-1 receptor; GPC, G protein complex; GPCRs, G protein-coupled receptor; JAKR, Janus kinase receptor; FASR, Fas receptor; IKK, IκB kinase; IκB, inhibitor of NF-κB; NF-κB, nuclear factor (NF)-κB; REL, REL protein; Ras, Ras protein; Rafs, Raf kinases; MAPK, mitogen-activated protein kinase; MEKs, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinases; MEF-2, myocyte enhancer factor-2; PPAK, family of p21-activated protein kinases; MSK, mitogen and stress activated protein kinase; MEK, mitogen-activated protein kinase; MAX, MAX protein; c-Myc, c-Myc protein; JUNFOS, Fos and Jun families of DNA binding proteins; Bcl-2, B-cell lymphoma 2; ELK-1, ETS transcription factor ELK1; AP-1, activator protein 1; ATF-2, activating transcription factor 2; PI3K, phosphoinositide 3-kinases; Akt, serine/threonine-protein kinases; mTOR, mammalian target of rapamycin; HIF-1α, hypoxia inducible factor 1 subunit alpha; VEGF, vascular endothelial growth factor; AMP, adenosine monophosphate activated protein; ATP, adenosine triphosphate; AMPK, AMP-activated protein kinase; p53, protein p53; PUMA, p53 upregulated modulator of apoptosis; NOXA, (PMAP1) – phorbol-12-myristate-13-acetate-induced protein 1; Bcl-xl, B-cell lymphoma-extra large; Bad, Bcl-2 associated death promoter; Bax, Bcl-2 associated protein x; Bid, BH3 interacting domain death agonist; STAT, signal transducer and activator of transcription; JAKs, Janus kinases; FADP, flavin adenine dinucleotide; cytc, cytochrome complex (187–192).

applied simultaneously to identify different Se compounds in plant extracts.

After the chromatographic separation, the mass of Se molecules can be detected by techniques such as the Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). These techniques detect Se molecules based on their transition ions which provide high accuracy detection, low detection limit (part per trillion), and less matrix interference (53, 54). The HPLC-ICP-MS has been considered a robust workflow and is widely used for Se determination in Se-containing plants and foods. A study by Ogra et al. (55) successfully applied size-excursion chromatography incorporated with ICP-MS to identify the Se metabolic pathway of ginger and Indian mustard using selenate or SeMet as Se fertilizers. The study found that γ-Glutamyl-Se-methylselenocysteine and MSC were the common metabolites of selenate and SeMet in garlic and Indian mustard.

As mentioned earlier, the Se compounds accumulated and stored differ by plant genus/species, and some Se can be bound to highly complex structure. In addition to the methods described above, other technique can be applied to identify the Se compounds started with compound purification by ion-exchange chromatography, followed by identification of the molecular mass by Electrospray Ionization-Mass Spectrometry (ESI-MS) (26, 56, 57). The ESI-MS is a technique that ionizes chemical compounds by electrospray ionization, and a mass analyzer then detects the ionized molecules according to their mass/charge (m/z) ratio (58). This high sensitivity mass spectroscopy technique can provide effective approaches to the speciation of Se bound in complex structures such as selenosugars and selenoproteins (59, 60). Some novel analysis methods have also been used to specify Se compounds in food materials. For example, Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry (LA-ICP-MS) is a solvent free analytical technique used to analyze Se compound in solid sample and it can provide greater accuracy results compared to traditional liquid chromatography (61). Moreover, the X-Ray Absorption Spectroscopy (XAS) technique was used to identify Se compounds in biological sample with less sample preparation step to prevent the degradation of Se compound

from chemical reaction during sample preparation (62). These analytical techniques can be valuable to identify any specific and new-found Se compounds in plants that could then be studied to understand their biological activity in the Se-enriched plant food products.

Bioactivity of Se compounds

Generally, literature shows that organic Se species tend to have higher bioactivities, bio-accessibility and lower toxicity than inorganic Se species. Research in human immortalized keratinocytes (HaCaT) cells showed that SeMet had a lower cytotoxicity effect on HaCaT cells than sodium selenite, where the IC_{50} of SeMet was 55.4 μM, much higher than 2.3 μM from sodium selenite (63). The lower cytotoxicity might be related to the antioxidant activity of organic Se compounds to prevent toxicity and cellular damage by increasing selenoamino acid and selenoproteins, which could enhance the activity of antioxidant enzymes such as glutathione peroxidase and thyroxine reductase (19, 64). For example, SeMet had increased GPx activity in rat skin cells at a higher dose than inorganic Se (selenite), which caused a toxic effect at 1μM (Hazane-Puch et al. (63). Moreover, SeMet increased the GPx activity and total antioxidant content while lower MDA formation in broiler chicken tissue compared to the sodium selenite-treated subjects (65).

On the other hand, the presence of Se compound in high concentration could generate cytotoxicity in human cells. Literature has identified several cytotoxic pathways of Se compounds across various human cancer cell lines (**Table 1**). Inorganic Se species, i.e., sodium selenite, was widely studied, especially on prostate cancer cells. The cellular toxicity mechanism of sodium selenite against human prostate cancer cells has been identified as below: generation of anti-proliferation effect via the expression of mRNA of the SELV, SELW, and TGR selenoproteins (66); promotion of GLS1 protein degradation and APC/C-CDH1 apoptosis pathways (67); induction of cell apoptosis via activation of caspase-8 protein (68); and activation of p53 protein (69). Moreover, the anti-proliferation activity of inorganic Se, including sodium selenite, has been reported in human lung cancer cell lines; it has involved inhibiting the Trx1 expression (70). Several signaling pathways are involved in cell anti-proliferation and apoptosis in human cells, as shown in **Figure 3**. Briefly, Se could cause cell death via apoptosis pathways by activating the executioner caspase-3, 6, 7, and 9, and promoting pro-apoptosis genes Bax and Bid on mitochondria and producing cytochrome C (CytC). The toxic effect of Se compounds could also mediate DNA repair and cell angiogenesis by promoting pro-apoptosis genes, including Bax and Bid (71).

A high concentration of Se compounds also performs a redox-active act as prooxidants, generating ROS in reaction (72). The redox action of Se compounds that generate ROS in the human cell could be the primary focus when using Se as an anticancer agent against human cancer cells. According to some studies (**Table 1**), SeMet could inhibit cell proliferation by inducing ROS generation and activating apoptosis cellular proteins, including the caspase family and p53 (73, 74). The ability to generate ROS could meditate the toxicity of Se due to the production of oxidative stress involved in cell cytotoxicity and apoptosis induction (75, 76). Moreover, MSC can induce cancer cell apoptosis via an interface of cell proliferation PI3K/Akt pathway (73), while $SeCys_2$ downregulated Bcl-2 survival genes in lung cancer cell lines (77). A study by Hui et al. (78) also showed that selenite induced cell apoptosis by upregulate cell death protein p38 MAPK and inhibition of the PKD1/CREB/Bcl-2 survival pathway.

The current research on Se compounds focuses on both sides of the spectrum: the protective effect against cell damage or the anti-proliferation effect against cancer cell lines. Se compounds' bioactive information could impact the functional properties of Se-enriched plant foods, not only the concentration of Se in the sample but also the chemical form of Se accumulated. Besides, the bioactive compounds such as polyphenol, polypeptides and polysaccharides in plant foods could also significantly affect the uniqueness of bioactivities and functional properties of Se enriched plants.

Biological properties of Se-enriched plant foods

The biological properties of Se-enriched plant foods have received more interest from researchers in the past two decades. **Figure 4** shows that the Se compound in Se-enriched plant foods induces biological activities through different metabolism pathways in human cells. Metabolism pathways of Se compounds begin with a reduction of inorganic or organic Se compounds from food supplements to hydrogen selenide (H_2Se). This H_2Se will be metabolized and synthesized into several selenoproteins, then transported and stored in human organs (79, 80). More than 25 selenoproteins have been identified in human cells, and some are considered antioxidant enzymes, such as glutathione peroxidase (GPxs), iodothyronine deiodinases, thioredoxin reductases (TrxR). These individual selenoproteins perform biological properties, including balancing plasma glucose levels and insulin sensitivities, anti-inflammatory and enhancing cell proliferation (4).

At their non-toxic concentration, Se-enriched plants could protect against cellular damage from hydrogen peroxide (H_2O_2) stress and enhance antioxidant enzymes in normal human cells. **Table 2** shows a compilation of research on the health effects of Se-enriched plants using *in vitro* human cells models. The antioxidant effect of Se-enriched food products has prevented oxidative stress induced by H_2O_2 in human cell lines. For

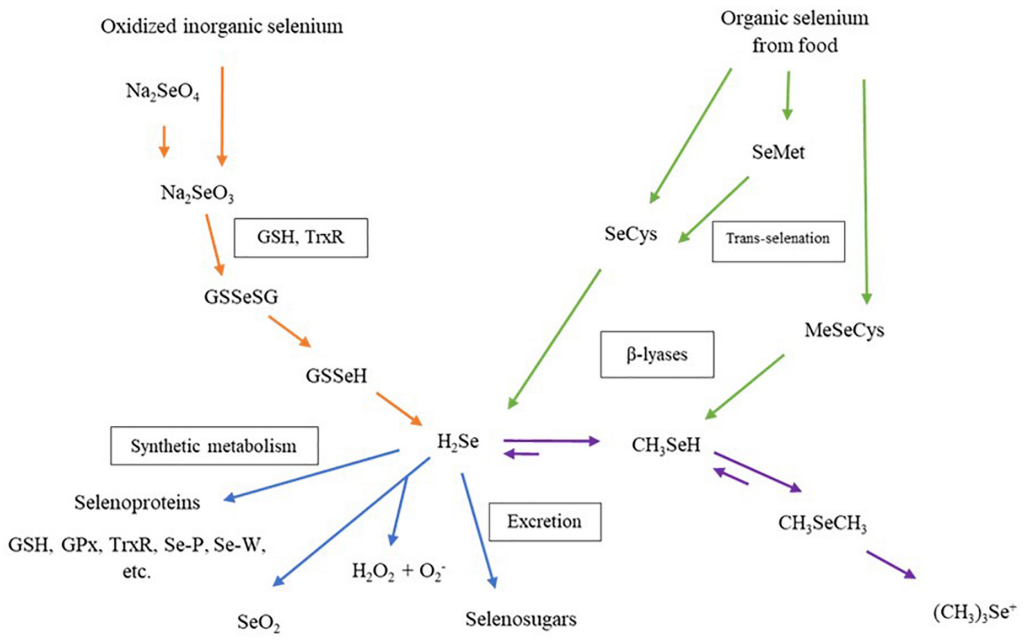

FIGURE 4
Metabolism of dietary selenium compounds in human cells. Na_2SeO_4, sodium selenate; Na_2SeO_3, sodium selenite; GSH, glutathione; TrxR, thioredoxin reductase; GSSeGS, selenodiglutathione; GSSeH, glutathioselenol; H_2Se, hydrogen selenide; GPx, glutathione peroxidase family; Se-P, selenoprotein P; Se-W, selenoprotein W; SeO_2, selenium dioxide; H_2O_2, hydrogen peroxide; SeCys, selenocysteine; SeMet, selenomethionine; MeSeCys, methylselenocysteine; CH3SeH, selenol; CH_3SeCH_3, dimethylselenide, $(CH_3)_3Se^+$, trimethylselenonium ion.

example, Se-enriched polysaccharides extracted from *Pleurotus ostreatus* and Se-enriched rice grass extract showed a protective effect against cellular oxidative stress from H_2O_2-induction in human muscle and human kidney cells (81, 82). Moreover, Se-enriched soybean peptide increased the activities of cellular antioxidant enzymes, including GPx, SOD, and CAT, in human colon cells (83, 84).

In contrast, Se-enriched plants could generate cellular ROS and influence cell death via the apoptosis mechanism at their toxic concentrations. For example, with human cancer cell lines, Se-konjac glucomannan performed anti-proliferation properties against human lung cancer cells (A549) and human breast cancer cells (HCC1937) by activating mitochondria pro-apoptosis protein caspase-3 (85). Furthermore, Se-enriched hawthorn fruit induced cellular apoptosis on human liver cancer (HepG2) cells by upregulation of pro-apoptosis protein caspase-9, downregulation of anti-apoptosis protein Blc-2, and increasing intracellular ROS level (86). These findings indicated that Se-enriched plant foods could perform both proliferation and anti-proliferation on either cancer or non-cancer cell lines and the effects depend on Se's dose and chemical forms in the diets.

Table 3 shows positive results on the biological properties of Se-enriched plants and some food ingredients (microalgae, probiotics bacteria and milk casein) in the *in vivo* animal models compared with Se-enriched yeast, an alternative source of SeMet (around 60-84%) with a lower toxic effect (87, 88). Various bioactive effects have been reported from Se-enriched plants, including increasing Se content in animal serum and tissue, enhancing antioxidant enzymes, lowering lipid oxidation in liver-stress animals, upregulation of cellular proliferation proteins, and downregulation of pro-inflammation and apoptosis cellular proteins. Some food products, for example, Se-enriched *Auricularia auricular* mushroom and Se-enriched radish sprouts, showed similar effects on improving antioxidant activities such as GPx and catalase, lower malondialdehyde (MDA) levels, and protecting liver damages in high-fat diet mice (89, 90). Se-polysaccharide from *Astragalus* also has anti-inflammatory effects on diabetic mice by lower serum inflammation-related proteins, including C-reactive protein, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and nuclear factor kappa B (NFκB) (91, 92). Moreover, Se-polysaccharide purified from *Pyracantha fortuneana,* and Se-enriched sweet potato inhibited tumor growth via apoptosis pathway and decreased IL-2, TNF-α, and VEGF in mice xenograft with human cancer tumor (93, 94).

In comparison, Se-enriched yeast (*Saccharomyces cerevisiae*) provides antioxidant and antitumor activities in animal studies with a lower affecting dose than Se-containing plants (95, 96). Se-enriched yeast could protect from oxidative stress and increase anti-inflammation by downregulating inflammatory cytokines such as TNF-a and NF-kB in aluminum-stress mice

TABLE 2 *In vitro* studies of Se-enriched plant foods against human cell lines.

Se-enriched food	Cell lines	Concentrations	Mechanisms/Pathways	References
Kale and kohlrabi sprouts	Human colon cancer cells (SW480, SW620), liver cancer cell (HepG2), uterus (SiHa) cells	1 mg ml^{-1}	Inhibit cell growth	(139)
Konjac glucomannan	Human lung cancer (A549), breast cancer (HCC1937) cells	0.15-0.6 μg ml^{-1}	↑Mitochondria apoptosis ↑Cleaved caspase-3 and ↑PARP-activated fragments	(140)
Polysaccharide from *Malus toringoides* (Rehd.) Hughes	Human liver cancer (HepG2) cells	50-200 μg ml^{-1}	↓ROS generation ↓H_2O_2 induction	(141)
Polysaccharide from alfalfa root	Human liver cancer (HepG2) cells	140 μg ml^{-1}	Inhibit cell growth	(142)
Soybean peptide	Human colon tumor cells (Caco-2)	10 μg ml^{-1}	↓ H_2O_2 induction ↑GPx; ↑SOD; and ↑CAT	(84)
Polysaccharide from *Tithonia diversifolia* (Hemsley) A.	Human gastric cancer (MKN7) cells	72.9-92.6 μg ml^{-1}	Inhibit cell growth	(143)
Hawthorn fruit	Human liver cancer (HepG2) cells	19.2 μg ml^{-1}	↑ROS generation ↑Caspase-9 ↓Blc-2	(86)
Broccoli sprout	Human prostate cancer (LNCaP) cells	0.27 μg ml^{-1}	↓PI3K/Akt/mTOR pathway	(144)
Polysaccharide from *Pleurotus ostreatus*	Human murine skeletal muscle (C_2C_{12}) cells	400 μg ml^{-1}	↓H_2O_2 generation Inhibit cell apoptosis	(82)
Ricegrass	Human kidney Cell (HEK293) cells	10 mg ml^{-1}	↓MDA ↓Oxidative stress and DNA damage	(81)
Kale roots	Human liver cancer (HepG2) cells	20 mg ml^{-1}	↑Nrf_2 protein	(145)
Se-enriched *Astragalus polysaccharide*	Human liver cancer (HepG2) cells	10 mg ml^{-1}	Inhibit cell growth	(146)
Polysaccharide from *Cordyceps gunnii*	Human ovarian cancer (SKOV3) cells	0.4 mg ml^{-1}	↑Cleavage caspase-3, -9, ↑PARP and ↑BAX ↓Bcl-2	(147)
Polysaccharide from *Rosa laevigata*	Human neuroblastoma (SH-SY5Y) cells	0.1 mg ml^{-1}	↓H_2O_2 generation	(148)
Polysaccharide from *Ginkgo biloba* L. leaves	Human bladder cancer (T24) cells	200 μg ml^{-1}	↑Cleavage caspase-3, -9, ↑PARP and ↑BAX ↓Bcl-2	(149)
Polysaccharide from *Pyracantha fortuneana*	Human breast cancer (MDA-MB-231) cells	400 μg ml^{-1}	Inhibit cell growth via cycle arrest at G2-phase ↑p53; ↑Bax; ↑Puma; ↑Noxa ↑Casepase-3,-9.↓Bcl2	(150)
Polysaccharide from *Pyracantha fortuneana*	Human ovarian cancer (SKOV3, HEY) cells	400 μg ml^{-1}	↑PARP; ↑Cleavage caspase-3; ↑Bax; ↓Bcl-2	(94)
Broccoli seed	Human Glioblastoma astrocytoma (U215) cells	28.5 μg ml^{-1}	Inhibit cell growth	(151)
Cauliflower	Human colon tumor (Caco-2) cells	2,500 μg ml^{-1}	Inhibit cell growth Changing cell morphology	(152)
Ziyang green tea	Human breast cancer (MCF-7) cells	172.2 μg ml^{-1}	Cycle arrest at G0/G1-phase ↑p53; ↑ Bax/Bcl-2 ratio; ↑caspase-3, -9; ↑ROS	(153)

↑, increase or upregulate; ↓, decrease or downregulate; Akt, protein kinase B; BAX, B-cell lymphoma 2 associated X; Blc-2, B-cell lymphoma 2; CAT, catalase; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; mTOR, mammalian target of rapamycin; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; Nrf2, nuclear factor erythroid 2–related factor 2; PARP, Poly (ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase; PUMA, p53 upregulated modulator of apoptosis; p53, tumor protein 53; ROS, reactive oxygen species; SOD; superoxide dismutase.

livers (97). The bioactivity of Se-enriched yeast could be due to the presence of SeMet as the main Se compound, where its biological properties have been widely studied. Compared to Se-enriched yeast, the bioactivity of Se-enriched plants is harder to explain and conclude. Not only because of the uniqueness of Se concentration and chemical forms in different plants, but the complexity of the food matrix also plays a significant role when studying the biological properties of Se-containing plant foods (4, 98). Food matrices, including protein and carbohydrates, can incorporate with Se via biosynthesis metabolism to form complex Se structures such as selenoprotein and selenopolysaccharide. The synthesized Se molecules can play a key role in the biological activity and bioavailability of Se-enriched food in humans (99). For instance, long-chain

TABLE 3 *In vivo* studies of Se-enriched plants and other food materials using animal models.

Se-enriched food or materials	Animal models	Treatment	Functional properties	References
Olive leaves	Growing rabbits	Treated with 2.17 mg Se kg^{-1} per dry leaves extract for 70 days	↑Serum antioxidant ↓Leukocyte DNA damage	(154)
Radish sprouts	CCl_4-induced liver injury mice	Treated with Se-enriched radish sprout in combination with inorganic Se compounds for 6 weeks	↓Inflammatory reaction in liver tissue ↓MDA in liver tissue ↑GPx in liver tissue	(90)
Gallic and cabbage	Broilers	Fed with a mixture of Se-gallic and cabbages	↑Se content in plasma ↑GPx in plasma	(155)
Garlic polysaccharide	Mice	Injected with 0.6 mg Se-polysaccharide	↑TNF-α; ↑IL-6; ↑IL-1 in macrophages	(156)
Radish sprout	Tumorigenesis induced rats	Treated with 12.5 ppm per day for 3 weeks	↑GPx; ↑GST in liver and lung	(157)
Kale spout	Male broilers	Treated with 2 mg Se kg^{-1} per day 42 days	↑Se content in animal tissue ↑GPx in plasma	(158)
Lotus leaf polysaccharide	Gestational Diabetes rats	Treated with 100 mg kg^{-1} per day for 7 weeks	↑GSH content, ↑GPx; ↑SOD; ↑CAT ↓FBG, ↓TG, triglyceride, LDL content.	(159)
Ziyang green tea polysaccharide	Chronic fatigue syndrome rats	Treated with 200 mg kg^{-1} per day for 4 weeks	↑Corticosterone ↓Aldosterone serum hormones	(160)
Rice	Diabetic mice	Treated with 0.2 mg g^{-1} body weight of 250 g L^{-1} Se-rice for 4 weeks	↓ C-reactive protein; ↓TNF-α; ↓IL-6; ↓COX-2 and ↑NFκB in serum	(91)
Wheat	Broilers	Treated with 37-185 μg Se kg^{-1} per day for 21 days	↑Se content in muscle and liver	(161)
Soybean peptide	Male Kunming mice	Treated at 4 mg Se kg^{-1} per day for 7 days	↑SOD in liver tissue ↓MDA in liver tissue	(84)
Soybeans	CCl_4-induced liver injury rats	Treated with 700 mg kg^{-1} twice a week for 8 weeks	↓α-SMA in the liver ↑mRNA expression of MMP9 ↑GSH; ↑GPx in liver tissue	(162)
Yellow pea and oat polysaccharides	Male weanling Sprague-Dawley rats	Treated with 40 μg Se kg^{-1} per day for 50 days	↑GPx in blood and liver ↑TrxR1 in liver	(163)
Soy protein isolate	Male weanling Sprague–Dawley rats	Treated with 30 μg Se kg^{-1} per day for 50 days	↑GPx in blood and liver ↑TrxR1 in liver	(164)
Auricularia auricular mushroom	High-Fat Diet Streptozotocin-induced diabetic mice	Treated at 500-1,000 mg kg^{-1} for 8 weeks	↓Diabetes-induced disorders of lipid metabolisms; ↓Liver damage ↑GPx; ↑CAT; ↓MDA in liver tissue	(89)
Grifola frondosa mushroom polysaccharide	Cyclophosphamide induced mice	Treated with 120 mg kg^{-1} per day for 7 days	↑GPx; ↑SOD; ↑CAT in serum, liver and kidney	(165)
Astragalus mushroom polysaccharide	CCl_4-induced liver injury rats	Treated with 40 mg per day for 7 weeks	↓TNF-α; ↓IL-6; ↓COX-2; ↓NFκB in liver tissue ↑Bcl-2/Bax ratio in liver tissue	(92)
Sweet potato polysaccharide	Hepatoma (H22) cells xenograft mice	Injected with 100 mg kg^{-1}	↑IL-2; ↑TNF-α; ↑VEGF in serum ↓Tumor growth ∼58%	(93)
Hypsizigus marmoreus polysaccharide	CCl_4-induced liver injury mice	Treated with 800 mg kg^{-1} per day for 10 days	↓MDA; ↓Lipid oxidation in serum and liver ↑GPx; ↑SOD in serum and liver	(166)
Pyracantha fortuneana polysaccharide	Human ovarian carcinoma (HEY) cells xenograft mice	Treated with 400 mg Se day^{-1} for 28 days	↓Cancer cell proliferation; ↑apoptosis ↓Cytoplasmic β-catenin	(94)
Pyracantha fortuneana polysaccharide	CCl_4-induced liver injury Kunming mice	Treated with 400 mg kg^{-1} per day for 5 weeks	↑GPx; ↑CAT in liver ↓TBAR; ↓ H2O2 in liver	(167)
Catathelasma ventricosum mycelia.	Streptozocin-induced diabetic mice	Treated with 500 mg kg^{-1} per day for 30 days	↑GPx; ↑SOD; ↑CAT; ↓ MDA in liver tissue	(168)
Agaricus bisporus mushroom	Hyperthermal induced oxidative stress rats	Treated with 1 μg Se g^{-1} per day for 5 weeks	↑GPx in *ex vivo* ileum	(169)

(Continued)

TABLE 3 (Continued)

Se-enriched food or materials	Animal models	Treatment	Functional properties	References
Pleurotus ostreatus mushrooms	Wistar male rats	Treated with 0.15 mg Se kg^{-1} per day for 21 days	↑Se content in plasma	(170)
Microalgae	Yearling common barbel fishes	Treated with 1 mg Se kg^{-1} per day for 6 weeks	↑GR in muscle and liver ↑Alanine aminotransferase; ↑Creatine kinase in blood plasma	(171)
Candida utilis	Sprague-Dawley rats	Treated with 3 mg Se kg^{-1} per day for 6 weeks	↑GPx; ↑SOD; ↑CAT; ↑GSH in serum and liver	(172)
Lactobacillus acidophilus	High-fat diet mice	Treated with 0.3 μg Se per day for 4 weeks	↑GPx; ↑SOD in serum ↓MDA; ↓TC; ↓TG; ↓LDL in serum	(173)
Lactobacillus acidophilus and Se-yeast	Crossbred weanling piglets	Treated with 0.46 mg Se kg^{-1} per day for 42 days	↑GPx in blood ↑TrxR mRNA in tissue	(174)
Lactobacillus acidophilus and Se-yeast	CCl_4-induced liver injury rats	Treated with 0.05 mg kg^{-1} Se per day for 7 weeks	↑GPx; ↑GSH; ↑SOD; ↓ MDA in liver tissue ↓ TNF-α; ↓IL-6; ↓MCP-1 in liver tissue	(175)
Milk casein isolate	Human epithelial breast cancer (MCF-7) cells xenograft mice	Treated with 1.15 μg Se g^{-1} per day for 70 days	↓Tumor volume ↑Apoptotic cells	(176)
Se-milk protein and yeast	Mice	Treated with 1 μg Se g^{-1} per day of either Se-milk protein or Se-yeast for 4 weeks	↑selenoprotein P; ↑GPx-2 in colon Only Se-yeast ↑GPx1	(177)
Se-yeast	Hepatotoxicity chickens	Treated at 50 μg kg^{-1} per day for 21 days	↓ALT; ↓AST; ↓MDA in serum ↑GPx; ↑SOD in serum	(178)
Se-yeast	Ochratoxin A-induced small intestinal injury chickens	Treated at 0.4 mg kg^{-1} per day for 21 days	↓Intestinal injury from ochratoxin A-induction via Nrf2 pathway ↓NF-κB activation	(179)
Se-yeast	5-fluorouracil induced mice	Treated with Se-yeast at 10^8 CFU per day	↓Eosinophil peroxidase activity; ↓CXCL1 levels; ↓Histopathological tissue damage ↓Oxidative stress.	(180)
Se-yeast	Aluminum exposed mice	Treated with 0.1 mg kg^{-1} per day for 28 days	↓Oxidative stress; ↓Inflammatory induction from Al-induction ↓mRNA inflammatory genes in liver tissue	(97)
Se-yeast	Mouse mammary tumor (EMT6) cells xenograft mice	Treated with 912 ng Se per day for 14 days	↓MDA in lung, brain, liver, thymus, spleen and kidney. ↑ Bcl-2; ↑p53; ↓IL-4 in tumor cells	(95)
Se-yeast	Yellow broilers	Treated with 0.15 mg Se kg^{-1} per day for 8 weeks	↑TrxR1; ↑GPx1 in kidney tissue	(96)

↑, increase or upregulate; ↓, decrease or downregulate; α-SMA, alpha-smooth muscle actin; ALT, glutamic pyruvic transaminase; AST, glutamic oxaloacetic transaminase; CAT, catalase; CCl_4, carbon tetrachloride; COX-2, cyclooxygenase-2; CXCL1, chemokine ligan-1; FBG, fast blood glucose, GSH, glutathione content; GST, glutathione S-transferases activity; GR, glutathione reductase; GPx, glutathione peroxidase activity; IFN-γ, interferon-gamma; IL-1, interleukein-1; IL-2, interleukein-2; IL-4, interleukein-4; IL-6, interleukein-6; LDL, low-density lipoproteins; MDA, malondialdehyde; MMP9, matrix metallopeptidase 9; MPC-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor erythroid 2–related factor 2; SOD, superoxide dismutase; TBAR, thiobarbituric acid reactive substances; TC, total cholesterol; TG, total triglyceride; TNF-α, tumor necrosis factor alpha; TrxR, thioredoxin reductase activity.

selenopeptide synthesized in soybean showed higher resistance in gastrointestinal digestion and lower toxicity risk compared with short-chain selenopeptide (100).

Clinical trials of selenium-enriched plant foods

Some beneficial properties of Se-enriched plant foods have been confirmed in *in vitro* cell models and *in vivo* animal studies. According to this evidence, there have been some human clinical trials performed to gain a robust understanding of the bioactivity of Se-enriched plant foods through the human metabolic system. **Table 4** presents a compilation of biological properties of Se-enriched plant foods and yeast as reported in human clinical trials. Improving the activity of antioxidant enzymes in human blood systems has been discovered as the primary biological activity of Se-containing plant materials. For example, Se-containing Brazil nuts have been found to enhance GPx activities and selenoprotein P

TABLE 4 Selenium-enriched plant foods and yeast human clinical trials.

Se-enriched food	Participants	Age group	Treatment	Functional properties	References
Onion	18 participants	50-64	50 μg Se daily for 12 weeks	↑T-cell proliferation after flu vaccination ↑IFN-γ; ↑IL-8; ↑Enzyme and perforin content in CD8 cells ↓TNF-α in CD8 cells	(105)
Broccoli	18 participants	24-65	200 μg Se per day for 3 days	↑Total Se level in plasma ↑Interleukin products in peripheral blood mononuclear cell	(106)
Rice	10 women participants	25 ± 2	80 g of Se-enriched rice (1.64 mg Se kg-1) per day for 20 days	↑Total Se level in plasma ↑GPx in plasma	(104)
Brazil nut	91 hypertensive and dyslipidaemia patients	62.1↑ ± ↑9.3	13 g of granulated Brazil nut (∼227.5 μg Se) per day for 12 weeks	↑Total Se level in plasma ↑GPx3; ↓oxidized LDL level in plasma	(102)
Brazil nut	89 dyslipidaemia and hypertensive patients	40-80	Brazil nuts 227.5 μg Se per day for 90 days	↓Total cholesterol; ↓non-HDL in serum Non-significantly different blood pressure and lipid content in serum	(181)
Brazil nut	81 hemodialysis patients	52 ± 15.2	5g Brazil nut (290.5 μg Se) per day for 3 months	↑Total Se level in plasma and erythrocyte ↑GPx in plasma	(103)
Brazil nut	61 participants	52-75	50 μg Se daily for 6 weeks	↑selenoprotein P; ↑β-catenin mRNA in blood Non-significantly decrease C-reactive protein in plasma	(101)
Se-yeast	36 polycystic ovary syndrome women	18-40	200 μg Se daily for 8 weeks	↓Cytokines IL-1; ↓TNF- α in serum ↑VEGF in serum	(182)
Se-yeast	491 participants	60-74	300 μg Se daily for 6 months and 2 years	↓Blood glucose marker hemoglobin at 6 months Non-significantly different at 2 years treatment	(107)
Se-yeast	400 participants	40-80	200 μg Se daily for 6 months	Non-significantly different in β-cell function or insulin sensitivity	(111)
Se-yeast	53 congestive heart failure patients	45-85	200 μg Se daily for 12 weeks	↑Insulin sensitivity index in serum ↓LDL; ↑HDL in serum	(108)
Se-yeast	80 lymphocytic thyroiditis patients	20-71	2 μg Se daily for 2 months, in combination with levothyroxine combined therapy	↑Therapeutic effect of levothyroxine ↓Thyroid-stimulating hormone; ↓Thyroid peroxidase antibody; ↓Thyroglobulin antibodies	(183)
Se-yeast	15 men	65-72.3	300 μg Se daily for 5 weeks	↓Epithelial-to-mesenchymal transition gene in Prostate biopsies	(184)
Se-yeast	76 participants	34.8	200 μg Se daily for 6 weeks	↓HbA1c gene refer to glycated hemoglobin in plasma Non-significantly fasten plasma glucose level	(185)
Se-yeast	60 diabetic patients	40-85	200 μg Se daily for 12 weeks	↓C-reactive protein; ↓matrix metalloproteinase-2; ↓MDA in plasma ↑Total plasma antioxidant capacity	(186)
Se-yeast	58 women with lipid profiles, plasma nitric oxide, or total antioxidant capacity conditions	18-55	200 μg Se daily for 6 weeks	↓Fasten plasma glucose level; ↓Serum insulin level; ↓Homeostasis model of assessment-insulin resistance ↓Triacylglycerol; ↑HDL level; ↑Total antioxidant capacity; ↑GSH in serum	(109)
Se-yeast	468 participants	60-74	300 μg Se daily for 5 years	↑Total Se level in plasma Non-significant different total cholesterol and HDL level in plasma	(112)
Se-enriched milk and Se-enriched yeast	20 participants	18-24	300 μg per day as Se-enriched yeast, and about 480 μg per day for Se-enriched milk for 8 weeks	Non-significantly different the plasma antioxidant enzyme	(113)

↑, increase or upregulate; ↓, decrease or downregulate; β-cell, beta-cells; CD8, cluster of differentiation-8; GPx, glutathione peroxidase activity; HbA1c, hemoglobin A1C; HDL, high-density lipoproteins; IFN-γ, interferon-gamma; IL-1, interlukein-1; IL-8, interlukein-8; LDL, low-density lipoproteins; MDA, malondialdehyde; T-cell, T-lymphocyte; TNF- α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

and lowering total cholesterol and LDL in older adults (101–103). Similarly, Se-enriched rice has been found to improve the total Se content and GPx activity in serum (104). Moreover, Se-enriched green onion and broccoli also showed beneficial effects in human clinical trials (105, 106). On the other hand, Se-enriched yeast has been applied as an effective and less toxic Se supplement to provide significant health properties. Se-enriched yeast could lower blood glucose, enhance insulin sensitivity, and lower the total cholesterol and LDL (107–109).

From these findings, Se-enriched plant foods at their non-toxic concentration can deliver health benefits by increasing antioxidant activity in human serum. Daily intake of Se for humans is about 55-70 μg Se per day, with the toxic level at 400 μg Se per day. From the data in **Table 4**, the dose of Se-enriched plant food and Se-enriched yeast in the range of 200-300 μg Se per day could provide health benefits without showing toxic side effects (110). The information from this review suggested that Se-enriched plant foods should be a safer choice for increasing dietary Se consumption due to a moderate concentration of Se in the plant investigated, and the organic Se compounds are significantly identified in plant food materials.

Overall, not many Se-enriched plants have successfully demonstrated a significant beneficial effect in human clinical trials (111–113) compared to the amount of investigations conducted in cell-based and animal models. Many factors can affect the results of clinical trials, including genetics, age, gender, ethnicity, personal behaviors, medical conditions, etc. (114, 115), and they need to be taken into account when designing a trial. It is essential to identify the bioactive compounds present in the plant materials, study how they can influence the bioactivity of the Se-enriched plant foods and verify the bioactivity and toxicity effects of the Se-enriched plant foods from the *in vitro* human cell lines and *in vivo* animal testing. All of these will provide information on the samples' biological properties, the corrective consumption level, and the toxicity dose of each Se-enriched plant food for human clinical trials.

Conclusion and future prospects

The biological properties of Se-containing plant foods are closely associated with the chemical forms and concentrations of Se content in the products. The studies on Se accumulation and speciation of Se compounds could provide helpful insight into the mechanism of Se-enriched plant foods' bioactivities. These beneficial bioactivities, including antioxidant, and anticancer properties of Se-enriched plant foods, have been positively demonstrated via *in vitro* human cell lines and *in vivo* animal studies. There is still a need for more human trials to relate the effect of Se-enriched foods and their health effects. Human clinical trials are critical to obtaining information regarding the consumption of Se-enriched food plants, considering different factors, including human genetic and age groups, and the effect of the food matrix.

Humans in different age groups (e.g., children, adults, elderly), gender, and health and physiological status (e.g., pregnancy and lactation) have different dietary requirements. Therefore, supplementing dietary Se to different groups of the population can be challenging as many factors need to be considered to ensure the supplementation deliver its intended health benefits. Due to the narrow gap between benefits and toxicity, precautions must be taken when considering Se enrichment in foods. The first thing to consider is the Se species present in the plant used for producing Se-enrichment foods. Since organic Se has far less toxicity, it is more suitable to be incorporated into food products. For safety reasons, it is essential to use Se-enriched plants that accumulate organic Se than those that accumulate a high inorganic Se content. Se-enriched plant foods with a moderate level of organic Se can be a more decent choice as a Se-supplement for all groups of people. Secondly, contamination from other metals, such as Cd and As, during Se accumulation can cause toxic stress in the plant and human health. Metal contamination in plants is mainly associated with the quality of soil and fertilizer applied during the enrichment stage. Soil quality and composition of Se fertilizer should be carefully monitored to avoid metal contamination of Se-enriched plants (116). Thirdly, limiting the consumption dose of Se-enriched food to a non-toxic level could prevent the harmful effect of Se toxicity. Regulations can be set and enforced to limit the level or serving size of Se-enriched foods to suit different groups of people. Furthermore, there is a need to establish suitable analytical methods to study Se speciation of various Se-enriched plant foods and perform more research to gather clinical information on bioactivity and toxicity when supplying Se-enriched plant food to different groups of the population. All these efforts are essential to protect from the negative effect of Se overdose, ensure safety and deliver the optimum benefit of Se-enriched foods to humans.

Future studies should cover the full spectrum of the research area, including identifying Se content and their chemical forms, in particular putting more effort on Se speciation of Se-enriched plant materials; screening their biological effects via *in vitro* assays or *in vivo* animal studies; and validating the findings in the human clinical trials. The evidence and knowledge from the above research could serve as a powerful motivation for the food industry to produce Se-enriched plant foods to combat Se deficiency and enhance life quality for the world population.

Author contributions

PT, JX, and SQ generated the presented idea in this manuscript. PT and SQ developed the theory, scope and performed the computation of the data. PT, PS, and SQ versified, analyzed, and discussed the collected data. All authors discussed the results and contributed to the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.962312/full#supplementary-material

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Higher serum selenium concentration is associated with lower risk of all-cause and cardiovascular mortality among individuals with chronic kidney disease: A population-based cohort study of NHANES

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Background: Selenium is an essential nutrient and trace element required for human health and plays an important role in antioxidative and anti-inflammatory processes. However, the long-term impact of selenium levels on the health of patients with chronic kidney disease remains unclear.

Method: Participants in this study were 3,063 CKD adults from the Third National Health and Nutrition Examination Survey (NHANES 1999–2000, 2003–2004, and 2011–2018). The mortality status and the cause of death of the study participants were obtained from the National Death Index records. For all-cause and cardiovascular disease (CVD) mortality, the models employed to estimate hazard ratios (HRs) and 95% CI were Cox proportional hazard models and competing risk models, respectively.

Result: During the follow-up period, 884 deaths occurred, including 336 heart-disease-associated deaths. The median (IQR) concentration of serum selenium was 181.7 (156.1, 201.5) μg/L. After full adjustment, serum selenium levels were associated with a decreased risk of mortality in patients with CKD, including all-cause and CVD mortality (P<0.001). The multivariate-adjusted HRs (95%CI) were 0.684 (0.549–0.852) for all-cause mortality ($P_{trend} < 0.001$) and 0.513 (0.356–0.739) for CVD mortality ($P_{trend} < 0.001$) when selenium concentrations were compared according to the extreme quartiles. Selenium levels are inversely associated with an increased risk of all-cause mortality and CVD mortality. Similar results were observed in subgroup and sensitivity analyses.

Conclusion: Higher serum selenium concentration was independently associated with a decreased risk of all-cause and CVD mortality in patients with CKD.

KEYWORDS

selenium, chronic kidney disease, antioxidant, oxidative stress, mortality

Introduction

Chronic kidney disease (CKD) is a serious health problem that poses a major threat to over 15–20% of the global general population and has become a significant challenge for society and healthcare systems worldwide (1). Among Medicare patients in the United States, the incidence of CKD has reached 14.5% and is higher in older adults (2–4). Patients with

CKD suffer from obviously high morbidity of comorbid cardiovascular diseases (CVDs), including arrhythmias and coronary artery disease (CAD) (1, 5). In the progression of CKD and comorbid CVDs, pro-inflammatory processes, oxidative stress, and vascular endothelial dysfunction can amplify and induce each other (6–8). Among the vicious circuits formed by pathological changes, oxidative stress can promote the development of chronic inflammation in patients with CKD and worsen renal injury (6, 9–13).

Selenium, an essential nutrient and trace element, plays a crucial role in anti-inflammatory and antioxidant processes (14). In the general population, meat and eggs are important dietary sources of selenium, whereas flour and rice are alternative dietary sources (15). Dietary selenium is absorbed in the intestinal tract and transformed into different metabolites through various metabolic pathways (16–18). Available dietary selenium is determined by the form of selenium (including organic and inorganic forms) and type of food (including meats, grains, and seafood) (16, 19). Insufficient dietary intake of selenium and selenium deficiency are important health challenges that induce Keshan disease, Kashin–Beck disease, autoimmune diseases, and CVDs (14, 20, 21). However, existing evidence reveals that high selenium levels have a beneficial impact on the incidence of various diseases in the general population, whereas supplementation with selenium has demonstrated controversial results (14, 22–33). Among patients with CKD with extremely high pro-inflammatory status and oxidative stress, who always have abnormal metabolisms of various trace elements, investigations regarding the beneficial effects of selenium are controversial and limited. One prospective study suggested that the serum selenium levels of patients with hemodialysis were similar to those of the general population (34), whereas three observational studies revealed that the serum selenium levels of patients with CKD were lower than those of healthy adults (28, 30, 32). Some intervention studies found that selenium supplementation may play a beneficial role in patients with CKD (35, 36), while a randomized controlled trial (RCT) indicated that oral administration of selenium does not decrease the prevalence of type 2 diabetes and leads to an increased risk of this disease during the follow-up period (37). To the best of our knowledge, no study has examined the long-term impact of serum selenium levels on all-cause mortality and CVD mortality in patients with CKD.

In the present study, we prospectively investigated the association of serum selenium levels with all-cause and CVD mortality among patients with CKD from the National Health and Nutrition Examination Survey (NHANES).

Methods

Study population

National Health and Nutrition Examination Survey (NHANES) is a nationally representative survey of the US civilian non-institutionalized population that is conducted by the National Center for Health Statistics (38). Since 1999, the NHANES has collected data continuously and released datasets every 2 years. The datasets contain information from personal interviews, physical examination results, and laboratory data. The NHANES cohort has been widely used to explore the associations between nutrients and mortality in the general population and different disease states (38–40). We conducted this cohort study using individuals from the NHANES 1999–2000, 2003–2004, and 2011–2018 cohorts with selenium measures and assessments of CKD.

Measurement of selenium levels and the diagnosis of chronic kidney diseases

As reported, the measurement method for serum selenium levels was inductively coupled plasma–dynamic reaction cell–mass spectrometry. In detail, the blood samples of participants were collected in containers, and then, the samples clotted and were centrifugated at 1,115 × *g* for 15 min. The serum samples were then stored under the proper freezing conditions and were prepared for transport to the laboratory. Based on the Kidney Disease Improving Global Outcomes (KDIGO) guideline, the estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio were used to define CKD (41). Using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, the eGFR of every participant was calculated. CKD was graded as follows: participants with eGFR $\geq$ 90 ml/min/1.73 m^2 and albuminuria were categorized into stage 1; participants with eGFR of 60–89 ml/min/1.73 m^2 and albuminuria were categorized into stage 2; participants with eGFR of 30–59 ml/min/1.73 m^2 were categorized into stage 3; participants with eGFR of 15–29 ml/min/1.73 m^2 were categorized into stage 4; and the participants with eGFR of $<$15 ml/min/1.73 m^2 were categorized into stage 5.

Mortality outcome of the study population

The source of mortality information was data collected from the National Death Index, until 31 December 2018. The follow-up time for each participant was examined from the time of participation to the date of death or 31 December 2018. Using the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10), the underlying cause of death was identified in this database. In the present study, we examined the effect of serum selenium levels on all-cause and CVD mortality. Specifically, codes I00–I09, I11, I13, and I20–I51 were defined as CVD deaths in ICD-10.

Covariates assessment

All data were obtained from the NHANES 1999–2000, 2003–2004, and 2011–2018 cohort databases. Demographic characteristics included in the present study were age, sex, race, family poverty-to-income ratio, body mass index (BMI), and smoking status. Serum triglyceride, total cholesterol, and uric acid concentrations were obtained from laboratory test results. Data on diabetes and hypertension were obtained as chronic comorbidities. Using total family income divided by the poverty threshold, the family poverty-to-income ratio was calculated. The smoking status

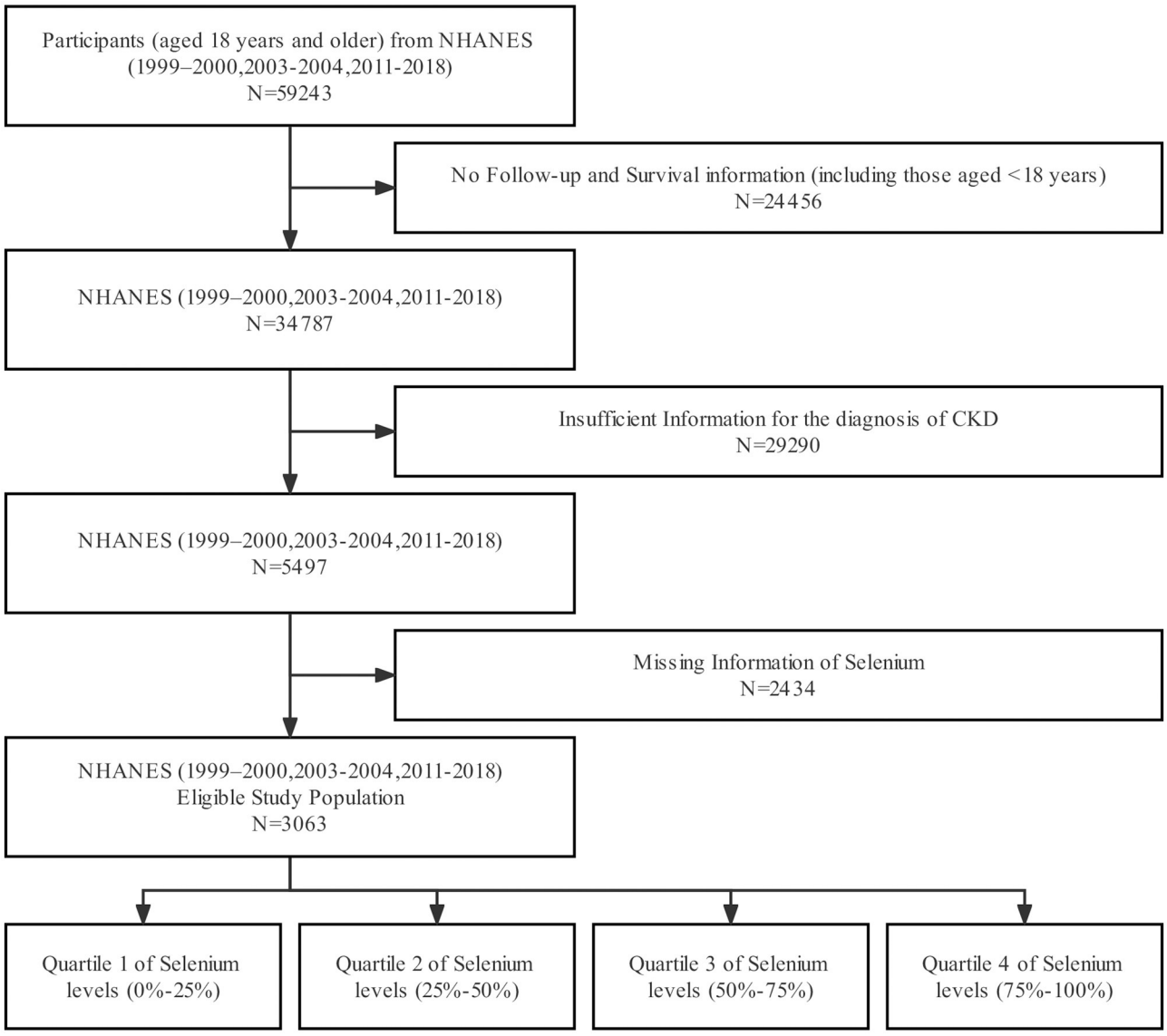

FIGURE 1
Flow chart.

of every participant was measured. The study population was categorized into smokers (smoking now or not smoking now but >100 cigarettes in life) and never smokers (<100 cigarettes in life). Diabetes was diagnosed according to a self-reported doctor's diagnosis of diabetes or laboratory test results. Positive laboratory test results included glycated hemoglobin (HbA1c) of ≥6.5%, fasting blood glucose of ≥7.1 mmol/L, random serum glucose of ≥11.1 mmol/L, and 2-h serum glucose of ≥11.1 mmol/L based on oral glucose tolerance tests.

Statistical analyses

Serum selenium concentrations were classified into four categories according to quartiles. Continuous variables are expressed as the mean (standard deviation), while categorical variables are expressed as numbers (proportions). Continuous and categorical demographic variables were compared using the analysis of variance (ANOVA) and the chi-square test.

First, we examined the correlation between serum selenium levels and mortality in patients with CKD using restricted cubic spline analyses. The Kaplan–Meier model analyses were conducted to estimate the cumulative incidence of all-cause death, whereas competing risk model analyses were employed to estimate the cumulative incidence of cardiovascular death. We examined the impact of different serum selenium levels on all-cause mortality using the Cox proportional hazards analysis models, whereas we investigated the impact of different serum selenium levels on cardiovascular-cause mortality using competing risk models. In model 1, the estimated models were adjusted for variables including age, sex, and race. In model 2, based on model 1, the estimated models were further adjusted for family income–poverty ratio,

TABLE 1 Baseline characteristics of participants with CKD according to serum selenium in NHANES (1999–2000, 2003–2004, and 2011–2018)[a].

Characteristics	Serum selenium concentration (μg/L)					
	Total	Q1 < 156.1	Q2 156.1–181.7	Q3 181.7–201.5	Q4 201.5–734.8	*P*
Number of patients, *n*	3,063	766	768	762	767	
Sex (%)						
Male	1,478 (48)	354 (46)	357 (46)	352 (46)	415 (54)	0.003
Female	1,585 (52)	412 (54)	411 (54)	410 (54)	352 (46)	
Age, years	64.0 (16.3)	70.0 (13.0)	63.5 (17.1)	61.5 (17.2)	60.9 (16.2)	<0.001
Ethnicity (%)						
Non-Hispanic white	1,455 (48)	449 (59)	331 (43)	327 (43)	348 (45)	<0.001
Other	1,608 (52)	317 (41)	437 (57)	435 (57)	419 (55)	
Family income–poverty ratio	2.3 (1.5)	2.3 (1.5)	2.2 (1.5)	2.2 (1.5)	2.4 (1.6)	0.148
Family income–poverty ratio (%)						
>3.0	896 (29)	222 (29)	226 (29)	209 (27)	239 (31)	0.11
1.1–3.0	1,478 (48)	396 (52)	354 (46)	373 (49)	355 (46)	
≤1.0	689 (22)	148 (19)	188 (24)	180 (24)	173 (23)	
BMI, kg/m^2	29.9 (7.1)	28.9 (6.5)	29.9 (7.5)	30.5 (7.3)	30.3 (7.2)	<0.001
Smoke status (%)						
Never smoker	1,516 (49)	348 (45)	384 (50)	403 (53)	381 (50)	0.034
Smoker	1,547 (51)	418 (55)	384 (50)	359 (47)	386 (50)	
Triglycerides, mg/dl	167.8 (128.3)	155.2 (123.8)	151.0 (101.7)	167.0 (116.9)	197.8 (158.9)	<0.001
Cholesterol, mg/dl	192.5 (46.8)	194.7 (46.6)	187.4 (44.0)	188.6 (45.0)	199.5 (50.4)	<0.001
Uric acid, mg/dl	6.0 (1.6)	6.1 (1.6)	6.0 (1.7)	5.9 (1.6)	6.1 (1.6)	0.111
Serum creatinine, mg/dl	1.2 (0.7)	1.2 (0.8)	1.2 (0.9)	1.1 (0.6)	1.1 (0.5)	<0.001
eGFR, mL/min/1.73m^2	70.2 (28.8)	62.2 (24.9)	69.0 (29.8)	72.6 (29.6)	76.9 (28.8)	<0.001
Urinary creatinine, mg/dl	117.1 (78.0)	112.2 (69.5)	119.6 (82.9)	115.0 (78.2)	121.5 (80.3)	0.078
Urinary albumin, μg/ml	201.2 (664.8)	202.8 (767.0)	226.0 (828.2)	202.2 (559.7)	173.8 (424.0)	0.498
Hypertension (%)						
No	836 (27)	178 (23)	226 (29)	224 (29)	208 (27)	0.02
Yes	2,227 (73)	588 (77)	542 (71)	538 (71)	559 (73)	
Diabetes (%)						
No	1,840 (60)	501 (65)	459 (60)	449 (59)	431 (56)	0.002
Yes	1,223 (40)	265 (35)	309 (40)	313 (41)	336 (44)	
CKD stage (%)						
Stage 1–2	1,543 (50)	304 (40)	374 (49)	410 (54)	455 (59)	<0.001
Stage 3–5	1,520 (50)	462 (60)	394 (51)	352 (46)	312 (41)	

[a]Continuous variables are presented as means (SD). Categorical variables are presented as numbers (percentages).
Q, quartile; BMI, body mass index; eGFR, estimated glomerular filtration rate; CKD, chronic kidney diseases.
No missing values.

BMI, serum triglycerides, serum total cholesterol, and serum uric acid. In model 3, based on model 2, the estimated models were the fully adjusted models, further adjusted for diabetes, hypertension, and smoking status. In model 4, serum selenium concentrations were analyzed as the continuous variable, and the results were fully adjusted for model 3. To confirm the previous correlation, age (≥65 or <65 years), sex (male or female), BMI (≥30 or <30 kg/m^2), race (non-Hispanic white or other), smoking status (smoker or never smoker), and various subgroup analyses were performed. Different sensitivity analyses were performed by excluding different subgroups of participants as follows: (1) participants who died within 1 year; (2) participants with CKD stages 3–5, and (3)

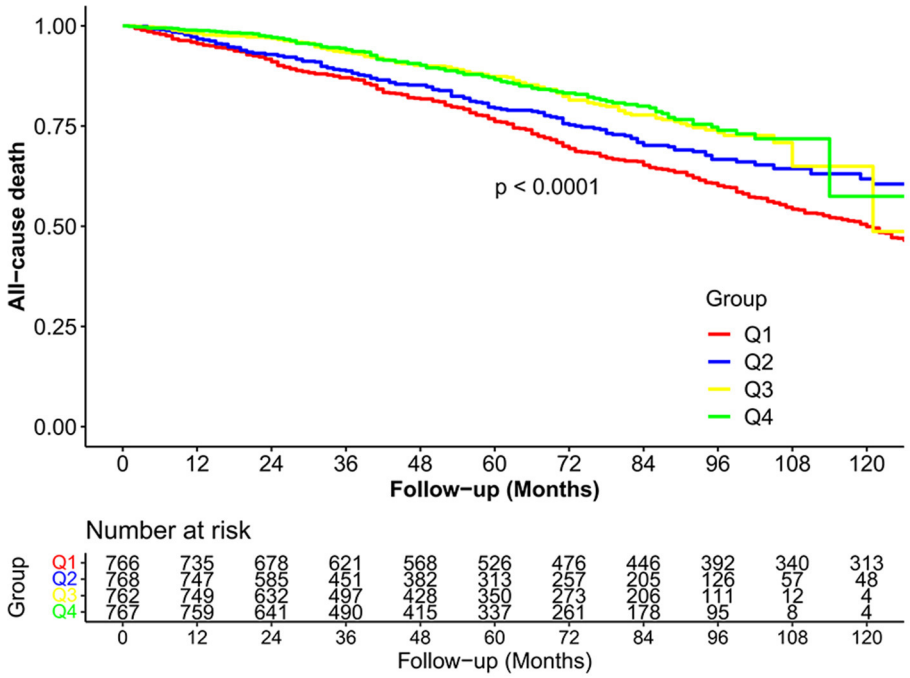

FIGURE 2
The cumulative incidence of all-cause death in the four study groups during the follow-up period.

participants with CKD stages 1–2. All statistical analyses were performed using R version 4.1.0.

Results

The current analysis included 3,063 individuals, and the complete preparation process is shown in Figure 1. The baseline characteristics of the study participants based on quartiles of serum selenium concentrations are presented in Table 1. In these participants, the median (interquartile range) serum selenium concentration was 181.7 (156.1–201.5) μg/L; the mean age was 64.0 ± 16.3 years, 48% were male, and 48% were non-Hispanic white. Higher serum selenium levels were associated with participants who were male, younger, of other races (different from non-Hispanic white), never smokers, and with a higher eGFR.

Of the 3,063 participants, during the follow-up period, 884 deaths occurred, including 336 cardiovascular deaths. The median follow-up period was 59 months. The Kaplan–Meier curve revealed that participants with higher serum selenium concentrations had significantly higher survival rates during the follow-up period ($P < 0.0001$; Figure 2). Similar results were observed between selenium concentrations and CVD mortality (Figure 3). The cumulative incidence of cardiovascular death in the different quartiles from Q1 to Q4 was 19.5, 17.5, 9.0, and 9.0%, respectively ($P < 0.001$), with a follow-up time of 120 months. Higher selenium levels were consistently correlated with a reduction in all-cause mortality among participants with CKD after multivariate adjustment (Table 2). As shown in Table 2, after multivariate adjustment, the hazard ratios (HRs; 95% CI) of the different quartiles from Q1 to Q4 were 1 (reference), 0.866 (0.724–1.035), 0.737 (0.596–0.911), and 0.684 (0.549–0.852), respectively ($P_{trend} < 0.001$). In model 4, all-cause mortality decreased by 0.5% for every 1% increase in serum selenium concentration (HR 0.995, 95% CI, 0.993–0.997; $P < 0.001$). A consistent correlation between selenium levels and all-cause mortality was observed according to the restricted cubic spline analysis results ($P < 0.001$; Figure 4A). A similar inverse correlation between serum selenium concentration and CVD mortality was observed, and higher serum selenium levels were correlated with a reduction in CVD mortality (Table 3). After multivariable adjustment, the HRs (95% CI) of the different quartiles from Q1 to Q4 were 1 (reference), 0.834 (0.633–1.100), 0.526 (0.370–0.748), and 0.513 (0.356–0.739; $P_{trend} < 0.001$), respectively. For every 1% increase in serum selenium concentration, the risk of CVD mortality decreased by 0.8% (HR 0.992, 95% CI, 0.989–0.996; $P < 0.001$). A non-linear dose–response relationship between serum selenium levels and CVD mortality was also determined ($P = 0.001$; Figure 4B).

When subgroup analyses were based on age, sex, race, BMI, and smoking status, a similar correlation was found between selenium levels and all-cause mortality (Table 4). We also investigated the correlation between selenium levels and CVD mortality using subgroup analysis (Table 5). When subgroup analyses were based on age, sex, race, BMI, and smoking status, the correlation of selenium levels with all-cause mortality and CVD mortality was unchanged.

After excluding the special participants from the sensitivity analyses, the analyses were repeated using a fully adjusted model (model 3; Supplementary Table 1). The correlation of serum selenium concentration with all-cause mortality and CVD

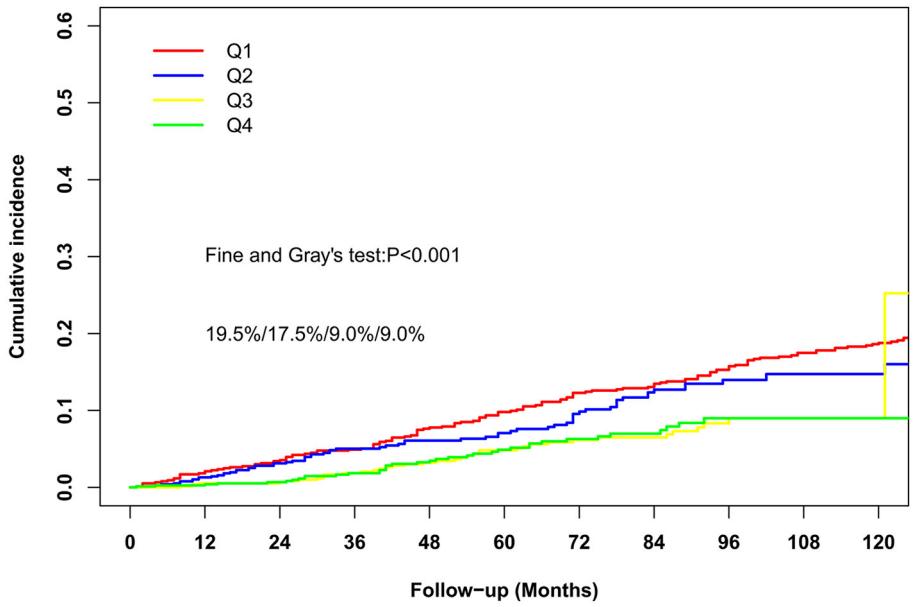

FIGURE 3
The cumulative incidence of cardiovascular-cause death in the four study groups during the follow-up period.

TABLE 2 All-cause mortality according to quartiles of serum selenium concentrations among patients with CKD[a].

Characteristics	Serum selenium concentration (μg/L)				*P*-trend
	Q1 < 156.1	Q2 156.1–181.7	Q3 181.7–201.5	Q4 201.5–734.8	
All-cause mortality					
Number of deaths/total	478/776	177/768	119/762	110/767	884/3,063
Model 1[b]	1	0.903 (0.756, 1.078)	0.748 (0.605, 0.924)	0.693 (0.557, 0.862)	$P < 0.001$
Model 2[c]	1	0.916 (0.766, 1.095)	0.753 (0.608, 0.931)	0.706 (0.566, 0.88)	$P < 0.001$
Model 3[d]	1	0.866 (0.724, 1.035)	0.737 (0.596, 0.911)	0.684 (0.549, 0.852)	$P < 0.001$
Model 4[e]	0.995 (0.993, 0.997)				$P < 0.001$

[a]Cox proportional hazards models were used to estimate the HRs (95% CIs) of all-cause mortality according to quartiles of serum selenium concentrations. Q, quartile.
[b]Model 1 was adjusted for age (continuous), sex (male or female), and race (non-Hispanic white or other).
[c]Model 2 was adjusted for age (continuous), sex (male or female), race (non-Hispanic white or other), family income–poverty ratio (>3.0, 1.1–3.0, and ≤1), BMI (≥30 or <30 kg/m^2), serum triglycerides (≥200 or <200 mg/dL), serum total cholesterol (≥240 or <240 mg/dL), and serum uric acid (≥7 or <7 mg/dL).
[d]Model 3 was adjusted for age (continuous), sex (male or female), race (non-Hispanic white or other), family income–poverty ratio (>3.0, 1.1–3.0, and ≤1), BMI (≥30 or <30 kg/m^2), serum triglycerides (≥200 or <200 mg/dL), serum total cholesterol (≥240 or <240 mg/dL), serum uric acid (≥7 or <7 mg/dL), diabetes (yes or no), hypertension (yes or no), and smoking status (smoker or never smoker).
[e]Continues model (each per 1% increase in serum selenium concentrations) adjusted by variables in model 3.

mortality was also unchanged after excluding participants who died in the 1st year during the follow-up period. Sensitivity analyses after excluding patients with CKD stages 1–2 or stages 3–5 indicated similar results.

Discussion

To the best of our knowledge, this is the first prospective study to investigate the correlation between serum selenium concentration and mortality among patients with CKD. In this study, we revealed a correlation between serum selenium levels and all-cause mortality and CVD mortality after multivariable adjustment for age, sex, serum triglyceride concentration, serum total cholesterol concentration, serum uric acid concentration, BMI, smoking status, hypertension, and diabetes. Based on the results of various analyses, we confirmed the reliability of these findings.

Selenium is an essential nutrient and trace element necessary for human health that plays a crucial role in antioxidative metabolism and homeostasis (42). Selenium compounds from daily intake include selenite, selenocysteine, selenomethionine, and methylselenocysteine, which have different metabolic pathways and metabolites (43–47). Selenium can prevent the formation of atherosclerotic lesions and improve endothelial function by reducing superoxide generation and preventing mitochondrial DNA damage (48–52). Epidemiological evidence also confirmed the health benefits of high serum selenium levels in reducing

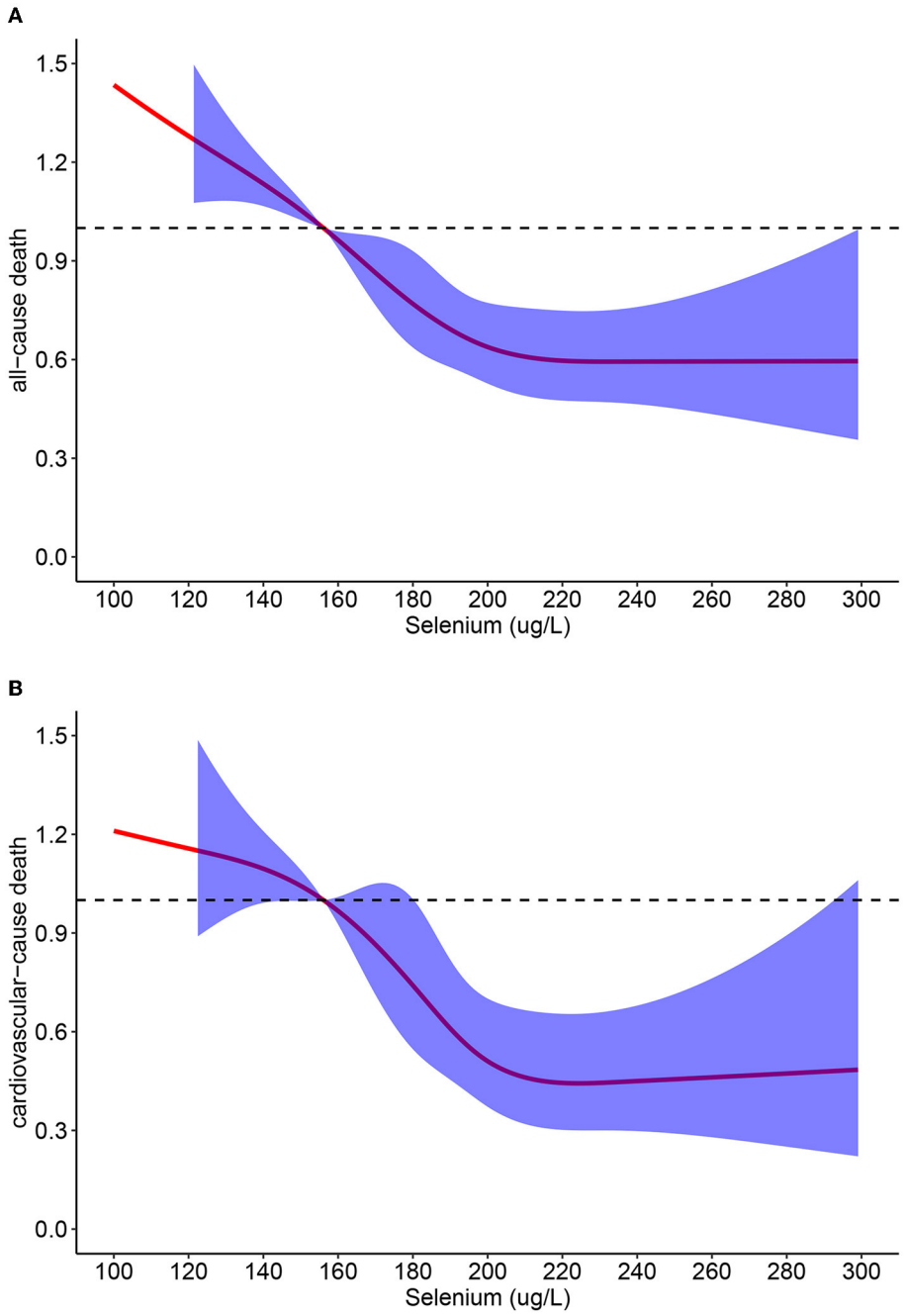

FIGURE 4
(A) Restricted cubic spline analyses between serum selenium concentrations and all-cause mortality; **(B)** Restricted cubic spline analyses between serum selenium concentrations and cardiovascular-cause mortality.

deaths and CVD events. Jayedi et al.'s dose–response meta-analysis supported that serum selenium levels and daily intake of selenium were inversely correlated with all-cause mortality (53). Kuria et al. found that high selenium concentration was associated with a decreased risk of CVD incidence and mortality, and a higher dietary intake of selenium was associated with decreased cancer risk after adjusting for age, BMI, and smoking status (54, 55).

In the current study, we found that serum selenium levels were lower in older participants among patients with CKD. This was similar to the results of the study by Schiavon et al., who revealed that serum selenium levels decreased years before death (56). Our results indicate that the correlation of serum selenium levels with all-cause mortality and CVD mortality was not associated with age. Similar results were obtained when subgroup analyses were based on sex and race. Smoking is an important risk factor for various types of progressive diseases and may significantly increase oxidative stress (57, 58), The results of the current study revealed that higher concentrations of selenium also significantly correlated

TABLE 3 CVD mortality according to quartiles of serum selenium concentrations among patients with CKD[a].

Characteristics	Serum selenium concentration (μg/L)				*P*-trend
	Q1 < 156.1	Q2 156.1–181.7	Q3 181.7–201.5	Q4 201.5–734.8	
CVD mortality					
Number of deaths/total	185/776	74/768	40/762	37/767	336/3,063
Model 1[b]	1	0.878 (0.670, 1.153)	0.560 (0.394, 0.794)	0.535 (0.371, 0.771)	$P < 0.001$
Model 2[c]	1	0.865 (0.657, 1.139)	0.540 (0.380, 0.767)	0.525 (0.363, 0.758)	$P < 0.001$
Model 3[d]	1	0.834 (0.633, 1.100)	0.526 (0.370, 0.748)	0.513 (0.356, 0.739)	$P < 0.001$
Model 4[e]	0.992 (0.989, 0.996)				$P < 0.001$

[a]Competing risk models were used to estimate the HRs (95% CIs) of cardiovascular-cause mortality according to quartiles of serum selenium concentrations. Q, quartile.
[b]Model 1 was adjusted for age (continuous), sex (male or female), and race (non-Hispanic white or other).
[c]Model 2 was adjusted for age (continuous), sex (male or female), race (non-Hispanic white or other), family income–poverty ratio (>3.0, 1.1–3.0, and ≤1), BMI (≥30 or <30 kg/m^2), serum triglycerides (≥200 or <200 mg/dL), serum total cholesterol (≥240 or <240 mg/dL), and serum uric acid (≥7 or <7 mg/dL).
[d]Model 2 was adjusted for age (continuous), sex (male or female), race (non-Hispanic white or other), family income–poverty ratio (>3.0, 1.1–3.0, and ≤1), BMI (≥30 or <30 kg/m^2), serum triglycerides (≥200 or <200 mg/dL), serum total cholesterol (≥240 or <240 mg/dL), serum uric acid (≥7 or <7 mg/dL), diabetes (yes or no), hypertension (yes or no), and smoking status (smoker or never smoker).
[e]Continues model (each per 1% increase in serum selenium concentrations) adjusted by variables in model 3.

TABLE 4 Subgroup analyses of the associations between serum selenium concentrations and all-cause mortality among CKD patients[a].

Characteristics	Serum selenium concentration (μg/L)				*P*-trend
	Q1 < 156.1	Q2 156.1–181.7	Q3 181.7–201.5	Q4 201.5–734.8	
Age, y					
<65	1	0.836 (0.523, 1.337)	0.605 (0.36, 1.015)	0.493 (0.296, 0.82)	0.003262
≥65	1	0.902 (0.742, 1.096)	0.798 (0.631, 1.008)	0.748 (0.585, 0.955)	0.007641
Sex					
Male	1	1.070 (0.846, 1.352)	0.718 (0.535, 0.964)	0.643 (0.478, 0.865)	0.00109
Female	1	0.675 (0.508, 0.897)	0.787 (0.576, 1.075)	0.797 (0.572, 1.111)	0.07929
Ethnicity (%)					
Non-Hispanic white	1	0.987 (0.786, 1.239)	0.739 (0.563, 0.97)	0.696 (0.529, 0.915)	0.00289
Other	1	0.701 (0.524, 0.937)	0.685 (0.485, 0.968)	0.644 (0.443, 0.936)	0.00813
BMI, kg/m^2					
<30	1	0.922 (0.739, 1.151)	0.727 (0.547, 0.967)	0.765 (0.58, 1.009)	0.014546
≥30	1	0.794 (0.585, 1.077)	0.738 (0.531, 1.025)	0.560 (0.389, 0.807)	0.00147
Smoking status					
Never smoker	1	1.024 (0.777, 1.347)	0.816 (0.589, 1.13)	0.738 (0.522, 1.045)	0.06053
Smoker	1	0.798 (0.63, 1.01)	0.704 (0.531, 0.933)	0.651 (0.49, 0.866)	0.000728

[a]Cox proportional hazard models were used to estimate the HRs (95% CIs) of all-cause mortality according to quartiles of serum selenium concentrations. Results were adjusted for age (≥65 or <65), sex (male or female), race (non-Hispanic white or other), BMI (≥30 or <30 kg/m^2), and smoking status (smoker or never smoker).
BMI, body mass index; Q, quartile.

with the reduction of all-cause mortality and CVD mortality in these patients. Similar results were obtained when subgroup analyses were based on BMI. CKD stage is a key risk factor for CVD mortality in patients (59, 60). Our results revealed that in both CKD stages 1–2 and 3–5, higher levels of serum selenium were correlated with a reduction in all-cause mortality and CVD mortality.

In the general population, the serum selenium concentration associated with minimal mortality is 130–150 μg/L (61, 62). However, there is an intrinsic conflict between long-term health benefits based on epidemiological evidence and the controversial results of selenium supplementation studies in humans (14, 24–33, 62). Countering this mystery, there are some possible explanations for this problem. First, in laboratory studies, selenite is the most common form of selenium, whereas most human studies use SeMet as the most popular supplement (14). This difference in chemical form may explain the differences in health effects between the studies. Second, the metabolites of cells and *in vitro* studies are different from those of *in vivo* studies and human studies

TABLE 5 Subgroup analyses of the associations between serum selenium concentrations and CVD mortality among CKD patients[a].

Characteristics	Serum selenium concentration (μg/L)				*P*-trend
	Q1 < 156.1	Q2 156.1-181.7	Q3 181.7-201.5	Q4 201.5-734.8	
Age, y					
<65	1	0.584 (0.283, 1.210)	0.466 (0.203, 1.070)	0.213 (0.080, 0.570)	<0.001
≥65	1	0.901 (0.669, 1.212)	0.599 (0.380, 0.822)	0.611 (0.415, 0.920)	<0.001
Sex					
Male	1	0.957 (0.664, 1.380)	0.491 (0.299, 0.806)	0.470 (0.284, 0.779)	<0.001
Female	1	0.667 (0.433, 1.028)	0.559 (0.336, 0.931)	0.588 (0.340, 1.018)	0.014
Ethic					
Non-Hispanic white	1	0.923 (0.652, 1.306)	0.489 (0.308, 0.777)	0.502 (0.317, 0.795)	<0.001
Other	1	0.694 (0.441, 1.092)	0.534 (0.305, 0.934)	0.482 (0.263, 0.884)	0.0064
BMI, kg/m^2					
<30	1	0.949 (0.672, 1.342)	0.541 (0.331, 0.886)	0.565 (0.344, 0.929)	0.0039
≥30	1	0.664 (0.422, 1.044)	0.492 (0.298, 0.811)	0.425 (0.247, 0.730)	<0.001
Smoking status					
Never smoker	1	0.922 (0.608, 1.399)	0.826 (0.515, 1.324)	0.488 (0.268, 0.892)	0.022
Smoker	1	0.791 (0.546, 1.147)	0.355 (0.208, 0.605)	0.531 (0.335, 0.841)	<0.001

[a]Competing risk models were used to estimate the HRs (95% CIs) of all-cause mortality according to quartiles of serum selenium concentrations. Results were adjusted for age (≥65 or <65), sex (male or female), race (non-Hispanic white or other), BMI (≥30 or <30 kg/m^2), and smoking status (smoker or never smoker).
BMI, body mass index; Q, quartile.

because of different environments and metabolic pathways. Third, excess selenium may harm normal glucose metabolic pathways and enhance the generation of peroxisome proliferator-activated receptor gamma (PPARγ) (63, 64). Therefore, previous studies have supported that those in the general population with serum selenium levels of 122 μg/L or higher may not need selenium supplementation (63). However, this is different for patients with CKD. Existing evidence indicates that serum selenium levels are lower in patients with CKD than those in the general population (28, 30, 31, 65). In the present study, we found that a higher concentration of serum selenium was correlated with decreased all-cause mortality and CVD mortality in patients with CKD. This is consistent with Ruiz et al.'s study, which found that adult patients on hemodialysis with lower selenium levels had a higher risk of death (65). In our study, the relationship between serum selenium levels and mortality among patients with CKD was different from the dual relationship confirmed in the general population. There are several potential explanations for this difference. First, the baseline levels of serum selenium and dietary intake of selenium in the two study populations were different (28, 30). Second, the oxidative stress levels in the two study populations were different, and abnormal oxidative stress levels were associated with abnormal selenium metabolism (1, 66). Third, serum selenium levels may not accurately reflect serum selenoprotein levels (14).

The present study has some strengths. First, the follow-up time of our study is long. Second, because the dietary intake of selenium in patients with CKD varied during the follow-up period, we used serum selenium levels as a better measure of selenium levels. Third, after adjusting for various covariates, the inverse correlation of selenium levels with all-cause mortality and CVD mortality was also evident, which confirmed the robustness of this association. Fourth, the subgroup analysis results indicated that the association existed generally in these subgroups of patients with CKD, which may reveal the universality of this association.

The present study has some limitations. First, although the current study was a cohort study, every participant's follow-up data were limited. Second, there was only one serum selenium concentration data point at the baseline, but the dietary intake of selenium changed gradually during the follow-up period, especially when the stages of CKD in patients progressed over years; thus, the baseline level of serum selenium may not accurately reflect the long-term level of serum selenium. Third, the dietary intake of selenium varies worldwide, and the level of serum selenium in our study participants was relatively high (14, 62). Thus, our results may not accurately reflect the association between selenium status and mortality in populations with lower serum selenium levels. Fourth, selenium's biological activities are mediated by selenium metabolites such as selenoproteins. Accurate measurement of selenoprotein and other selenium metabolites will reveal the underlying metabolism between serum selenium and mortality. Finally, the correlation is not causation; therefore, our results cannot prove causality because of the inherent limitations of observational studies.

Conclusion

Higher serum selenium concentration was independently associated with a decreased risk of all-cause mortality and CVD mortality in patients with CKD.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

This study involved secondary data analysis of a nationally representative publicly available dataset. The study we conducted was exempted from institutional review for this reason.

Author contributions

DZ and QZ analyzed the data and drafted the manuscript. TL and TS designed the study and revised the manuscript. All authors approved the final version of the manuscript, ensured the accuracy, integrity of the study, and agree to be accountable for all aspects of the study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1127188/full#supplementary-material

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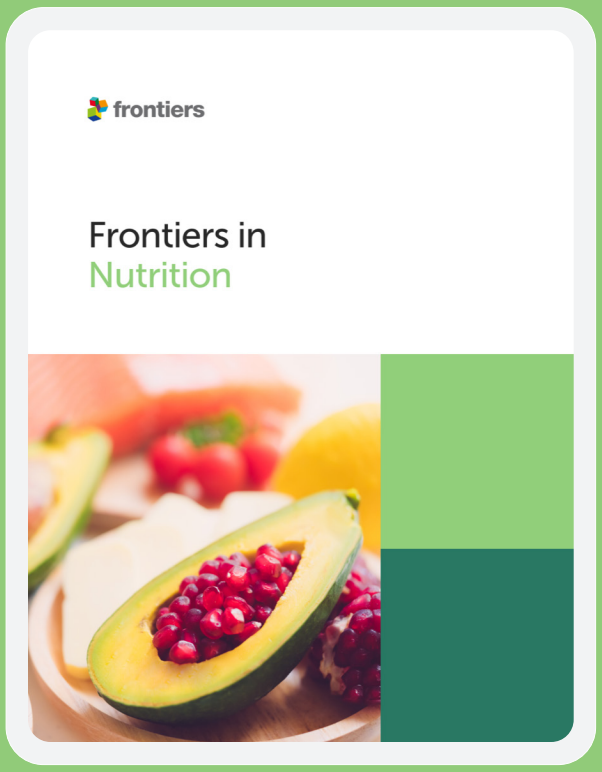

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