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Bioaccessible selenium sourced from Se-rich mustard cake facilitates protection from TBHP induced cytotoxicity in melanoma cells

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Abstract

Selenium (Se) is an essential dietary supplement that resolves inflammatory responses and offers anti-oxidant cytoprotection. In this study, we present data on the cytoprotective effect of Se-rich mustard protein isolated from mustard cultivated in seleniferous soils in Punjab, India. The concentration of total Se in mustard seed, oil free mustard cake, and mustard protein were 110.0 ± 3.04 , 143.0 ± 5.18 and 582.3 ± 6.23 $\mu\text{g/g}$, respectively. The cytoprotective effect of Se-rich mustard protein was studied on tert-butyl hydroperoxide (TBHP)-induced cytotoxicity in a mouse melanoma cell line (B16-F10). When compared with TBHP treated cells (where no viable cells were found), Se-rich protein made bioaccessible through *simulated* gastrointestinal digestion, protected melanoma cells from cytotoxicity with decreased levels of oxidative stress resulting in 73% cell viability. Such an effect was associated with a significant increase in glutathione peroxidase activity as a function of bioaccessible Se and its response towards cytoprotection.

Graphical abstract

Introduction

Selenium (Se) is an essential micronutrient for humans, animals, and some microorganisms. The nutritional importance of Se associated with various biological functions such as anti-inflammatory, thyroid metabolism, antioxidant defense and immune functions^{1–3} is now well established. Se deficiency has been linked to many clinical disorders such as viral infection, increased risk of cancer, various neurologic diseases such as Parkinson's and Alzheimer's disease, a decrease in thyroid and immune functioning, and male infertility.⁴ In livestock animals, deficiency of Se leads to major health consequences such as mulberry heart disease, retained placenta in ruminants, white muscle disease, pancreatic fibrosis, and fertility disorders.⁵ These Se deficiency diseases can effectively be prevented by supplementation in animal diets.⁶ Many studies have already demonstrated the benefits of Se supplementation, particularly in deficient populations, in the alleviation of human health disorders.^{7–10}

Se, upon absorption, is incorporated co-translationally into the 21st amino acid selenocysteine (Sec), into 25 selenoproteins in humans that include glutathione peroxidases (GPx), thioredoxin reductases (Txnrd), and iodothyronine deiodinases (DIOs), selenoprotein P (SelenoP; SePP), MsrB1, SelenoK, SelenoF, SelenoT, SelenoH, SelenoO, SelenoM, SelenoN, SelenoI, SelenoS, SelenoV, SelenoW, and other proteins.¹¹ These selenoproteins protect cells from free radical-induced oxidative damage, in addition to playing an important role in redox signalling, regulation of thyroid hormone synthesis, calcium homeostasis, prevention of hydroxide-induced ferroptosis.^{12–14} The Se levels in blood plasma accounts for the up-regulation of selenoenzyme-gene expression in body and therefore is directly dependent on Se intake. Plants are the major dietary source of Se through the food chain for humans, and Se content of plants is mainly dependent on the soil where they are cultivated. Plants uptake the Se from the soil in the form of selenate, selenite or as organic Se species.¹⁵ Following uptake, Se is incorporated into organic compounds in plants using various metabolic pathways similar to their respective sulfur analogs.¹⁶ Human or animal consumption of plant-based diets leads to the incorporation of Se into selenoproteins as well

as varying degrees of non-specific incorporation as selenomethionine (SeMet), depending on the amount of methionine available in the diet.

Indian mustard (*Brassica juncea*) is well known as a good source of essential nutrients both to humans and livestock. According to the USDA Food Composition Database (USDA-02024), 100 g mustard seeds contain approximately 28 g carbohydrate, 26 g protein, 36 g total fat, 12 g dietary fiber and 0% cholesterol. They also contain a trace amount of vitamins, electrolytes (Na and K) and minerals essential to human and animals, such as Ca, Cu, Fe, Mg, Mn, Se, and Zn. This species, when cultivated in agricultural soils naturally impacted by Se in the Nawanshahr region, Punjab (India), has been observed to potentially accumulate significant levels of Se in parts of the plant.^{17,18} Generally, due to its high nutritional value, the mustard cake is prominently used as a supplement for animal feed, but consumption of high-Se mustard feed confirmed Se poisoning in live stocks.¹⁹ Alternatively, the Se-rich mustard cake can be effectively utilized as Se supplement to livestock in Se-deficient regions. In addition, the possibility of Se-rich proteins from mustard being used as supplements for Se-deficient human population could also be useful. However, there are no studies to justify the benefits of such a fortified food source in animals or humans.

Keeping this in view, we aimed at determining (a) the levels of Se in protein extract obtained from Se rich mustard seeds; (b) the role of bioaccessible Se in peroxide-induced cytotoxicity in mouse melanoma (B16-F10) cells; and (c) activity of cellular GPx in these murine melanoma cells. Our studies suggest that bioaccessible Se in mustard seeds effectively protect cells from peroxide toxicity by inducing cellular GPx activity thus making a strong case for translation using *in-vivo* models.

Materials and Methods

The post-harvested seeds of Indian mustard (*Brassica juncea*, cv. SEJ-2) were collected from seleniferous region (Se-impacted) at a site near the village of Jainpur geographically located at 31° 13' N, 76° 21' E, in the Nawanshahr district of Punjab State (India). Seeds from non-seleniferous (non-impacted) mustard crop were collected from Patiala district (30° 34' N, 76° 38' E), Punjab, which is located about 75 miles away from the seleniferous region.

Approximately 10 kg of collected mustard seeds (from both Se-impacted and non-impacted regions) were used for oil extraction through a screw oil expeller. Chunks of oil cakes were dried overnight at 40° C, ground into fine powder and further deffated with hexane to remove residual oil. Se was analyzed in mustard seed, oil, and cake powder using fluorescence spectrometric method¹⁸. This method involved digestion of samples with HNO₃ and HClO₄, reduction of Se from Se⁺⁶ to Se⁺⁴ with HCl, complexing of Se⁺⁴ with 2,3-diaminonaphthalene to form piazselenol and extraction of the piazselenol in cyclohexane. The emission spectrum of piazselenol complex was measured using fluorescence spectrometer (Perkin Elmer LS-45, USA) at excitation and emission wavelengths of 360 and 520 nm, respectively [Detection limit: 2ng/ml]. Se quantification in each sample was carried out by relative method using emission spectrum of National Institute of Standards and Technology (NIST) certified Se-ICP standard solution (SRM-1349). Storage protein from mustard cake powder was extracted with distilled water

and precipitated with chilled acetone. Total Se content in isolated protein was further determined as described.²⁰

To increase the cellular bioaccessibility of Se, isolated protein was subjected to simulated gastrointestinal (GI) digestion using pepsin and pancreatin.²¹ In short, 500mg of both Se-rich and normal mustard protein was transferred separately into 50 ml Erlenmeyer flasks, each containing 10 ml of gastric juice solution (6% w/v pepsin in 0.15 M NaCl, adjusted to pH = 1.8 with HCl). Initially, the mixture was shaken vigorously for 1-2 min and then placed in shaker-incubator at 37° C with gentle shaking (50 rpm) for 3 h. pH of the solution was then adjusted to 7.0 with saturated solution of NaHCO₃. After adding 10 ml of intestinal juice solution (mixture of 2% w/v pancreatin, 0.2% bile salt in 0.15 M NaCl), flasks were degassed and further incubated for 4 h at 37° C with gentle shaking. The digests were then centrifuged at 8000 rpm at 4° C for 10 min and filtered. Before treatment to the cells, the digests were incubated at 90° C in water bath for 1 h to inactivate the enzymes and filtered. The digests were then subjected to Se determination. Protein isolated from non-seleniferous mustard was taken as control.

The cytoprotective effect of GI digested Se-rich mustard protein (GI-Se) was performed against tert-butyl hydroperoxide (TBHP) on mouse melanoma cell lines (B16-F10). Sodium selenite (Na₂SeO₃) was used as positive control, whereas undigested Se-rich (uGI-Se), undigested non-seleniferous (uGI-nSe) and GI digested non-seleniferous (GI-nSe) mustard protein fractions were used as corresponding samples for comparison. The sample for undigested protein was prepared by dissolving 500 mg of protein powder into 20 ml of phosphate buffer saline (PBS), followed by centrifugation and Se quantitation.

B16-F10 cells are a murine tumor cell line commonly used as a research model for human skin cancers and to study the metastasis and solid tumor formation.^{22,23} Briefly, B16-F10 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin. Prior to Se treatment, a total of 1.0×10^4 cells was seeded in collagen precoated 96 well tissue culture plate. The plate was then incubated in 5% CO₂ containing humidified air at 37° C. After 70% confluence, culture medium was replaced with a fresh medium containing 1 µM of Se (as sodium selenite or Se-rich protein) and further incubated for 24 h. Each well was washed twice with PBS and a fresh medium was added containing 2.0 mM TBHP, followed by additional 12 h incubation. Se-induced cytoprotection against TBHP was assessed using a standard MTT assay and cell staining within the same 96 well plate. After TBHP treatment, live and dead cells were differentiated by neutral red and trypan blue, respectively. For staining purposes, cells in the last well of each column in culture plate were washed twice with PBS and 100 µl of neutral red dye (40 µg/ml, in DMEM) was added to each well followed by incubation for 1 h at appropriate culture conditions. Wells were then washed twice with PBS, followed by addition of 20 µl trypan blue solution (0.4% w/v) per well incubation for another 10 min. Each well was washed once with PBS and 100 µl of PBS was added to each well before microscopic imaging.

Cytoprotective effect of the Se-rich mustard protein against TBHP is expected to be due to diverse variety of defensive mechanisms including increased expression of Se-dependent

glutathione peroxidase (GPx). Present study was also focused on Se-dependent assessment of the levels of GPx in mouse melanoma cells. A total of 1.0×10^6 cells was seeded in collagen precoated 90 mm tissue culture plates containing DMEM growth medium with composition mentioned earlier. After 70% confluence, growth media was replaced with a fresh medium containing 1 μ M of Se from different sources (Na_2SeO_3 ; uGI-Se; GI-Se) whereas, GI-nSe was used as a negative control as described in MTT assay and plates were incubated for 24 h. Cells were washed twice with PBS free of Ca^{2+} and Mg^{2+} and harvested by scraping. Scraped cells were resuspended into 1.0 ml of 50 mM phosphate buffer (pH 7.8), sonicated on ice for 2 min (pulse time 9 sec) and centrifuged at 15,000 rpm, 4° C for 30 min. Cell homogenates were transferred into 1.5 ml microfuge tube and stored at -20° C until assayed. Total protein concentration in cell homogenates was estimated by Bradford method using bovine serum albumin as standard. Glutathione peroxidase activity was determined according to the method given by Weydert and Cullen.²⁴ Briefly, 1.0 ml of disposable polystyrene cuvette containing 900 μ l of GPx assay solution (50 mM potassium phosphate buffer (pH 7.0), 1.0 mM Na_2EDTA , 1.0 mM NaN_3 , 1.0 U/ml glutathione reductase, 1.0 mM reduced glutathione (GSH) and 0.2 mM NADPH) and cell homogenate containing 50 μ g of protein was mixed together and incubated for 5 min at 37° C. Reaction was started after addition of 10 μ l of H_2O_2 (2.5 mM) followed by measuring the change in absorbance at 340 nm for 5 min. Glutathione peroxidase enzyme (0.02-0.1 U/ml) was used as standard to calculate the GPx activities in samples.

Experiments were carried in triplicate, where ever applicable, and were expressed as mean $\pm$ SD values. The comparison between seleniferous and non-seleniferous samples was performed using student t-test ($P < 0.05$) with Graph pad prism v5.0.

Results and Discussion

As a Se accumulator, mustard plant, cultivated on Se impacted soils, can also accumulate significantly high levels of Se in their seeds. Table 1 presents the levels of Se in both non-seleniferous and Se-rich mustard samples as determined by fluorescence spectrometer.

Total Se content analyzed by fluorescence spectroscopic method in the non-seleniferous and Se-rich mustard seeds were 0.87 ± 0.10 μ g/g and 105.4 ± 1.54 μ g/g, respectively; whereas in the defatted mustard cake, it was found to be 1.18 ± 0.06 μ g/g and 138.90 ± 1.6 μ g/g, respectively. A very small fraction of Se was also detected in Se-rich mustard oil (2.60 ± 0.29 μ g/g), but it was below detection limit in control oil. A significant difference ($P < 0.001$) in Se levels were observed in all mustard samples when Se-rich samples compared with their respective controls.

Most of the plants possess the ability to absorb, metabolize, and store a significant amount of Se in their tissues during their growth on Se-contaminated soils. Se content in soil varies from low (0.03-0.08 μ g/g) to toxic ranges and toxic (seleniferous) levels can extend up to 1200 μ g/g.²⁵ In India, particularly in the Nawanshahr region of Punjab, a vast agricultural land (> 1000 hectares or ~ 2500 acres) are severely affected with high levels of Se ranging from 0.25 to 4.55 μ g/g with a mean value of 3.63 μ g/g.²⁶ Previous studies carried out by our research group, using instrumental neutron activation analysis, indicated the soil Se to be in

the range of 2.7-6.5 µg/g and Se in different crop produce (wheat, rice, maize, and mustard) to be in the range of 13-670 µg/g.^{17,18,21} Plants of the families *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Lecythidaceae*, *Fabaceae*, *Rubiaceae*, and *Schrophulariaceae* have a tendency to accumulate high levels of Se (1000-10,000 µg/g dry weight) are categorized as Se hyperaccumulators. These plants accumulate Se, 100-fold higher than those of surrounding vegetation without displaying any apparent symptoms of toxicity.¹⁶ The Se hyperaccumulator plants primarily biosynthesize non-protein selenoamino acids, such as methyl-selenocysteine (MeSeCys) and γ-glutamyl-methyl-selenocysteine (γ-Glu-MeSeCys) that prevent the damaging effect on plant functions resulting upon incorporation of Sec and SeMet.²⁷

Indian mustard accumulates large amounts of Se as noted in the present study and also supported by other observations, indicating rapid translocation of Se from root to shoot.²⁸ Once Se is taken up by plant roots in a particular chemical form, whether organic or inorganic, it undergoes metabolic changes that determine the final product as well as its translocation and accumulation in different plant tissues.²⁹ A study on the comparison of Se levels in different plant organs of hyperaccumulator plants, including those of *Brassica* species, suggest that Se is actively transported through the plant in favorable growth conditions.³⁰ As the leaves mature, their Se levels decline, which could be a result of dilution as the leaves expand, and also due to export to reproductive tissues, such as seeds, which contain some of the highest levels in the entire plant.³⁰

Isolated protein was subjected to quantification of Se and total Se content in Se-rich protein was 582.3 ± 6.23 µg/g; whereas Se concentration in non-Se mustard protein was below detection limit of instrument (2 ng/ml). Cytoprotective effect of the Se-rich protein against TBHP induced toxicity in B16-F10 cells was monitored by differential cell staining and MTT assay. TBHP is an organic hydroperoxide that causes alteration to the redox state of the cell, lipid peroxidation, DNA damage and ultimately induces *in-vitro* cellular oxidative stress.³¹⁻³³

MTT assay results clearly indicated no cytotoxicity in the cells when treated with Se as Na₂SeO₃ or as uGI-nSe/uGI-Se (figure 1B1, C1, D1 and F1). However, without Se pretreatment or when the cells were treated with non-Se mustard protein, TBHP induced complete cell death (figure 1A2, C2 and D2), demonstrating that non-Se mustard protein does not exert any cytoprotective effect against TBHP. Cells pre-treated with Na₂SeO₃ showed 84% (figure 1B2), followed by 73% in presence of GI-Se (figure 1F2) and 47% with uGI-Se (figure 1E2). Se available in digested or undigested protein was notably less accessible than Se as sodium selenite. Between the GI digested and undigested samples, Se was observably more bioaccessible in GI digested form as demonstrated by 73% viability in the former compared to 47% in the latter. Therefore, it is presumed that GI-enzymes hydrolyze the proteins into amino acids and small peptides, which can be easily absorbed by the cells when compared to intact proteins.

In mustard seeds, methionine is found as major sulfur-containing amino acid³⁴ and due to the identical chemistry of sulfur with Se, it is expected that Se-rich mustard proteins also contains SeMet along with methionine. Previous reports on biofortification of mustard with

Se have shown that after enzymatic hydrolysis of seed or oil-free meal, SeMet was the major selenoamino acid.^{35,36}

The protective role of Se from oxidative damage by free radicals is well documented in the literature. The unregulated production of free radicals inside the cell has been related to various oxidative stress diseases.³⁷ Expression of selenoproteins in cells and plasma are critical in the protection from insults affected by reactive oxygen and nitrogen species from the oxidation of LDL, which is important in the regulation of plasma cholesterol and prevention of atherosclerosis and myocardial ischemia.^{38–41}

Glutathione peroxidase (GPx) family of selenoproteins serve as an essential component of cellular detoxification system that protect the cells from free radical-induced oxidative stress, including ferroptosis as shown recently in the case of GPx4.¹⁴ In the present studies, significantly high GPx activity was demonstrated in mouse melanoma cells treated with GI digested Se-rich protein showing nearly similar effect to that of sodium selenite. The treatments of Se were similar as described in MTT assay but without TBHP. Following various treatments, cell homogenates were used to estimate changes in their GPx activity. Figure 2 presents the GPx activity in melanoma cells (expressed in U/mg of protein).

The DMEM medium used for the culture of melanoma cells were Se deficient as seen from the low GPx activities observed in control and non-Se mustard protein treated cells. However, Se supplementation significantly enhanced GPx expression. The GPx activities in Se treated cells (Na₂SeO₃, uGI-Se and GI-Se) were significantly higher ($P<0.05$) w.r.t control and the fold increase in GPx activities were 2.51, 1.38 and 2.31, respectively. No significant difference ($P>0.05$) was observed in GPx activity of the non-Se mustard protein (GI-nSe) treated cells, when compared to control. Gpx activity also varied with the nature of Se treatments. The cells treated with Na₂SeO₃ expressed significantly higher ($P<0.05$) GPx activity when compared to GI-digested and undigested Se-rich mustard protein. As expected, Se-dependent increase in GPx activity (1.38-2.51 folds) were cytoprotective, particularly upon exposure to oxidative stress in the form of high concentrations of TBHP (2 mM) as showed by the MTT assay.

Literature is replete with studies showing the impact of added Se on Se-dependent GPx expression in mammalian cells as function of bioaccessible levels of Se. Kayanoki et al.⁴² reported that under Se deficient conditions, lower doses of H₂O₂ exhibited apoptotic cell death by internucleosomal DNA fragmentation in bovine renal epithelial cell lines, when compared to their Se supplemented counterparts. As seen in the current studies, decreased expression of selenoproteins, under Se deficient conditions, suggested hierarchical degradation of some selenoprotein mRNA such as GPx1 that is significantly down regulated as seen in other *in-vitro* cell systems.^{43–45}

Present *ex-vivo* studies indicated that Se bioaccessibility from inorganic Se species was preferred over Se-rich protein, whereas, many *in-vivo* studies on animal models have shown evidence for higher Se uptake from organic sources.^{46–48} However, Se sourced through biological or biocompatible organic matrices facilitate slow and sustained release as well as its steady incorporation of Se into selenoprotein synthesis, as demonstrated by various

research groups and reviewed by Bodner et al.⁴⁹ This aspect is, therefore, a salient feature of the use of Se-rich mustard protein over the inorganic Se in form of sodium selenite.

In conclusion, our studies demonstrate the significant localization of Se in the protein fraction of mustard and its cytoprotective activity towards TBHP-induced oxidative stress in mouse melanoma cells. In comparison with undigested mustard protein, enzymatic digestion of protein enhanced the bioaccessibility of Se and reduced the oxidative stress in these cells. Simultaneously, the activity of GPx enzyme was also observed to be enhanced as a function of bioaccessible Se. These results suggest that high levels of bioaccessible Se in mustard proteins from Se-rich mustard can serve as a good source of Se to enhance antioxidant activity in mammalian cells. It will be interesting to explore if diets rich in Se-mustard can accord the same level of protection from oxidative stress-related injuries in rodent models to enable future clinical trials in humans.

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Figure 1.

Representative microscopic images (400x) of stained mouse melanoma cells with treatments of Se (1μM) and TBHP (2mM) along with their percentage cell viabilities: (A1) only growth medium, (A2) TBHP, (B1) Se as sodium selenite, (B2) pretreatment with Se as sodium selenite + TBHP, (C1) undigested non-Se mustard protein, (C2) pretreatment with undigested non-Se mustard protein + TBHP (D1) GI digested non-Se mustard protein, (D2) pretreatment with GI digested non-Se mustard protein + TBHP, (E1) Se as undigested Se-rich mustard protein, (E2) pretreatment with Se as undigested Se-rich mustard protein + TBHP, (F1) Se as GI digested Se-rich mustard protein, (F2) Pretreatment with Se as GI digested Se-rich mustard protein + TBHP.

Figure 2.

GPx activities in melanoma cells after various treatments with Se after 24h.

Se supplementation was 1 μ M as sodium selenite or as Se rich protein. The observations were significant difference between each of the treatments (n=3) i.e., *** at $P < 0.05$, except in the case of control vs GI-nSe where there was no significant difference (t-test). (GI-nSe: undigested non-Se mustard protein; uGI-Se: undigested Se-rich mustard protein; GI-Se: GI digested Se-rich mustard protein)

Table 1

Total Se content in non-seleniferous and Se-rich mustard samples analyzed by fluorescence spectrometer (n=3; mean $\pm$ SD)

Sample	Total Selenium ($\mu\text{g/g}$)	
	Non-seleniferous	Selenium-rich
Mustard seed	0.87 ± 0.10	105.4 ± 1.54
Mustard oil	BDL	2.60 ± 0.29
Mustard cake	1.18 ± 0.06	138.90 ± 1.6

BDL- below detection limit (2ng/ml)