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Selenoneine ameliorates peroxide-induced oxidative stress in *C. elegans*

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ABSTRACT

Scope: Selenoneine (2-selenyl-N_α, N_α, N_α-trimethyl-L-histidine), the selenium (Se) analogue of the ubiquitous thiol compound and putative antioxidant ergothioneine, is the major organic selenium species in several marine fish species. Although its antioxidant efficacy has been proposed, selenoneine has been poorly characterized, preventing conclusions on its possible beneficial health effects.
Methods and results: Treatment of *Caenorhabditis elegans* (*C. elegans*) with selenoneine for 18 h attenuated the induction of reactive oxygen and nitrogen species (RONS). However, the effect was not immediate, occurring 48 h post-treatment. Total Se and Se speciation analysis revealed that selenoneine was efficiently taken up and present in its original form directly after treatment, with no metabolic transformations observed. 48 h post-treatment, total Se in worms was slightly higher compared to controls and no selenoneine could be detected.
Conclusion: The protective effect of selenoneine may not be attributed to the presence of the compound itself, but rather to the activation of molecular mechanisms with consequences at more protracted time points.

Selenoneine (2-selenyl-N_α, N_α, N_α-trimethyl-L-histidine) is the predominant form of organic selenium (Se) in tuna, swordfish and other marine fish species [1–3]. Being the Se analogue of ergothioneine, a sulfur-containing putative antioxidant [4] it has attracted considerable attention and similar beneficial properties have been attributed to selenoneine. However, data are still insufficient. In an *in vitro* radical scavenging assay, selenoneine was more effective as compared to ergothioneine towards 1,1-diphenyl-2-picrylhydrazyl [1]. It was also hypothesized to bind to heme proteins, preventing iron auto-oxidation [1,5]. Moreover, a reduction of methylmercury (MeHg) accumulation and toxicity in zebrafish embryos was reported in the presence of selenoneine [6]. The proposed mechanism of detoxification was posited to involve the formation of selenoneine-MeHg complexes and their excretion *via* the ergothioneine transporter OCTN-1, which was also discussed in dolphin liver [7]. Recently, feeding a selenoneine-containing tuna extract was shown to reduce the pathology of experimental colorectal cancers in mice, and the authors suggested this extract 'might be effective as a dietary antioxidant' [8]. The detection of selenoneine and its methylated derivative Se-methylselenoneine in human blood and urine demonstrates its bioavailability and putative metabolism in humans [9–11]. However, the pure compound has yet to be assessed *in vivo*, and the interplay between bioavailability, metabolism and possible protective effects, as well as underlying mechanisms have yet to be deciphered.

In the present study, we addressed these endpoints in the well-characterized *in vivo* model *Caenorhabditis elegans* (*C. elegans*). The nematode provides many intrinsic advantages as an alternative and complementary model organism, including an invariant and fully described developmental program and a high degree of evolutionarily conserved genes and signaling pathways [12]. Moreover, its rapid life cycle allows studying effects during the course of the worms' development with minimum effort. Recently, immediate and sustained effects of selenomethionine (SeMet), Se-methylselenocysteine (MeSeCys) and selenite on the antioxidant defense system have been studied in *C.*

Abbreviations: C. elegans, *Caenorhabditis elegans*; ESI-Orbitrap-MS, electrospray ionization orbitrap mass spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; MeSeCys, Se-methylselenocysteine; OCTN-1, organic cation / carnitine transporter 1; QQQ, triple quadrupole; RONS, reactive oxygen and nitrogen species; RP-HPLC, reversed phase high performance liquid chromatography; SeMet, selenomethionine; *t*-BOOH, *tert*-butyl-hydroperoxide

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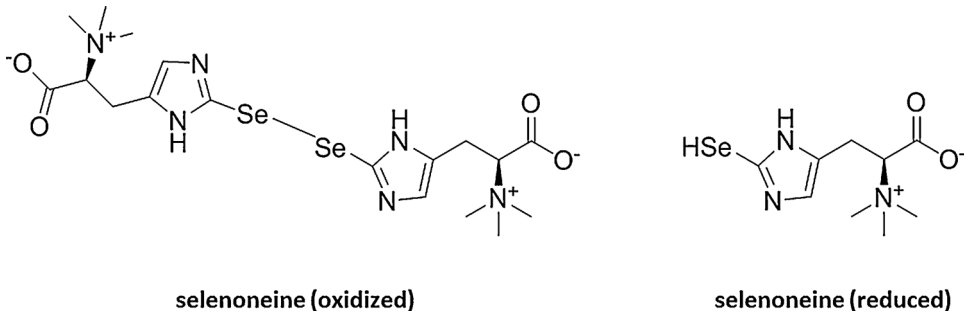

Fig. 1. Chemical structures of selenoneine in its reduced monomeric form as well as its oxidized dimeric form.

elegans, and their protective efficacy towards chemically induced formation of reactive oxygen and nitrogen species (RONS) has been demonstrated [13]. Based on the previously established experimental design [13], synchronized wildtype *C. elegans* (N2, *Caenorhabditis* Genetics Center, Minneapolis, USA) were exposed to selenoneine for 18 h during hatching. Subsequent endpoint analysis was conducted either directly post-treatment in L1 larvae, or 48 h post-treatment in worms grown to L4 stage without additional selenoneine exposure to investigate possible sustained effects. Selenoneine was isolated and purified in its dimeric form (Fig. 1) from genetically modified yeast *Schizosaccharomyces pombe* as described elsewhere [14]. Stock solutions (10 mM) were prepared in purified water, stored at −80 °C and diluted shortly before each use. Eggs were hatched in M9 buffer supplied with 1 or 10 µM selenoneine, or M9 buffer only. Although in the previous study, 100 µM of the Se species were used, the lower concentrations were chosen to mimic an exposure scenario that is closer to the physiological situation, with respect to uptake and respective exposure scenarios.

Following incubation, L1 stage larvae were exposed to *tert*-butyl-hydroperoxide (*t*-BOOH), and RONS formation was measured in intact worms using the 5(&6)-carboxy-2',7'-dichloro-dihydrofluorescein-diacetate (carboxy-DCFH-DA) assay. This assay was established and optimized regarding the applied *t*-BOOH and carboxy-DCFH-DA concentrations for both L1 and L4 stage worms as extensively described in the previous study [13]. Interestingly, immediately after selenoneine treatment, the extent of RONS induction was indistinguishable between control worms and selenoneine-treated worms, hence, no protective effect occurred at this time point (Fig. 2A). However, 48 h post-treatment, a pronounced, dose-dependent attenuation of *t*-BOOH induced RONS formation was observed (Fig. 2B). The absence of RONS-attenuating effects directly following incubation is in contrast to our previous study, since the therein tested Se species effectively diminished *t*-BOOH induced RONS formation immediately post-treatment, as well as 48 h post-treatment [13]. This discrepancy may arise from the lower selenoneine concentrations applied here, as opposed to the 10- to 100-fold higher doses used previously, but might also be driven by the different Se species. In general, L1 stage worms command a highly potent antioxidant defense system to protect the developing nematodes, which are more vulnerable to exogenous stressors due to their immature, penetrable cuticles. Therefore, a much higher *t*-BOOH concentration was necessary to induce RONS in this early life stage as compared to L4 stage worms (L1 stage: 350 µM *t*-BOOH, L4 stage: 50 µM *t*-BOOH; selenoneine provided at 1 and 10 µM). Given the fact that the oxidant concentration was multiple times higher as compared to the applied selenoneine concentrations, along with the powerful antioxidant system at base level, the selenoneine treatment might be unable to contribute to any additional benefits at this developmental stage. Interestingly, 48 h post-treatment, selenoneine was more effective in diminishing RONS levels as compared to higher doses of other Se compounds. For instance, treatment with 1 µM selenoneine attenuated RONS formation to a similar extent as 100 µM MeSeCys after 120 min, and had an approximately 20–30% more pronounced quenching effect

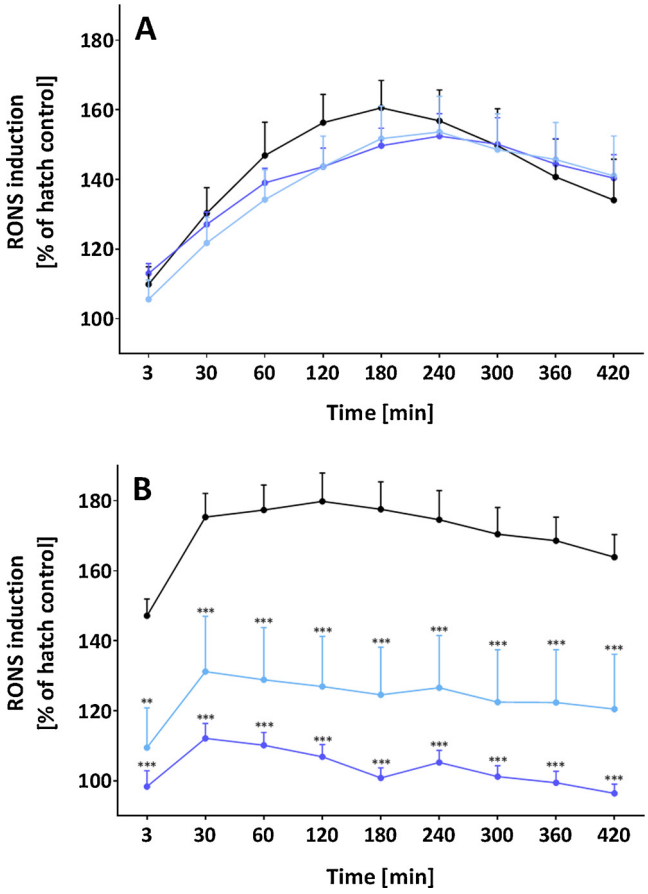

Fig. 2. RONS induction by *tert*-butyl-hydroperoxide (*t*-BOOH) following exposure to selenoneine for 18 h during hatching measured after dye loading and subsequent treatment with 350 µM (L1) or 50 µM (L4) *t*-BOOH. Data were normalized to the corresponding negative control (w/o *t*-BOOH) of each hatch treatment group. Shown are mean values of at least two independent experiments measured in triplicate wells + SEM. Statistical analysis by two-way ANOVA, followed by Dunnett's *post hoc* test: *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* control. (A) L1 stage. (B) L4 stage (48 h post-treatment). Mean fluorescence intensity of control worms (w/o *t*-BOOH) after 3 min: 3000 units at L1 stage, 7000 units at L4 stage.
control, 1 µM selenoneine, 10 µM selenoneine.

than 100 µM selenite or SeMet [13]. Compared to the lower dose, 10 µM selenoneine was even more effective and inhibited *t*-BOOH induced RONS formation almost completely.

In order to obtain mechanistic insights for the observed effects, we investigated the bioavailability as well as the retention of selenoneine in the worms. Thus, total Se contents were quantified immediately and 48 h post-treatment *via* isotope dilution ICP-QQQ-MS following microwave-assisted acid digestion according to protocols published before

Table 1
Total selenium (Se) and selenoneine contents of *C. elegans* following hatch incubation (18 h) with selenoneine, directly after treatment (L1 stage, 0 h) and 48 h post-treatment (L4 stage). Shown are values of at least two independent experiments.

	0 h post-treatment (L1 stage)		48 h post-treatment (L4 stage)	
Applied concentration	**ng Se/mg protein** *(x-fold of control[a])*	**ng selenoneine/mg protein**	**ng Se/mg protein** *(x-fold of control[b])*	**Selenoneine [µg selenoneine/L]**
1 µM	4.0 – 4.3 *(4.5 – 4.7 x)*	1.4 – 2.3	1.3 – 1.8 *(1.1 – 1.5 x)*	< 1
10 µM	33.2 – 33.9 *(36.9 – 37.7 x)*	13.8 – 25.6	1.7 – 2.2 *(1.4 – 1.9 x)*	< 1

[a] 0.8 – 1.0 ng Se/mg protein (L1 stage).
[b] 0.9 – 1.4 ng Se/mg protein (L4 stage).

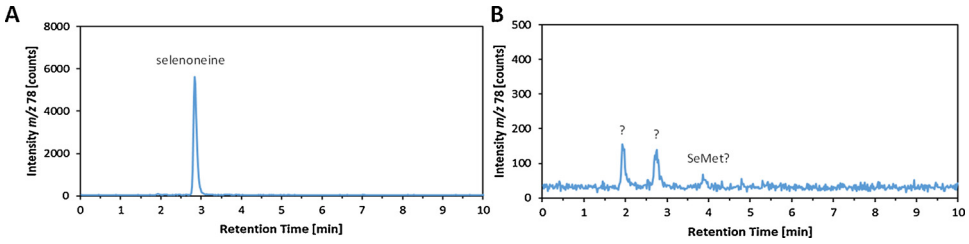

Fig. 3. RP-HPLC-ICP-MS elution profiles of *C. elegans* lysates following hatch incubation (18 h) with 10 µM selenoneine, (A) directly after treatment or (B) 48 h post-treatment; column: Atlantis dC18 (4.6 x 150 mm), 20 mM ammonium formate 0.1 mM TCEP 3% MeOH pH 3; 1 mL/min; injection volume: 10 µL.

[13]. As shown by the dose-dependent increase in total Se at L1 stage, selenoneine was effectively taken up by the worms (Table 1). In addition, to investigate if selenoneine is metabolized in *C. elegans*, Se speciation analysis was carried out. Thus, worms were pelletized directly after or 48 h post-treatment, purified water was added to a total volume of 250 µL, and lysates were obtained by sonication and centrifugation as previously described [15]. An aliquot was subjected to protein determination *via* the bicinchoninic acid (BCA) assay, and after a second centrifugation step (20 000 x g, 4 °C, 20 min), RP-HPLC-ICP-MS was performed according to a previously published method [16]. Se speciation analysis revealed that the major part of Se was present in the form of selenoneine at L1 stage (Table 1, Fig. 3A). The presence of selenoneine was confirmed by HPLC-ESI-Orbitrap-MS as described before [16], while no additional small organic Se species were detected. Hence, no indications of selenoneine metabolism were obtained. The difference between selenoneine and the total Se content (Table 1) might have been caused by the fact that due to the low amount of sample, total Se determination and Se speciation analysis were performed with different sets of specimens. Moreover, the missing Se might be bound or partially attached to proteins or peptides, thus requiring further investigation. 48 h post-treatment, total Se contents in worms hatched in the presence of selenoneine were only slightly higher compared to untreated controls (10 µM *vs.* control: $p = 0.0510$), indicating that most of the ingested compound is excreted until L4 stage. Accordingly, selenoneine was neither detectable by RP-HPLC-ICP-MS (Table 1, Fig. 3B) nor by HPLC-ESI-Orbitrap-MS. Traces of an unknown Se species eluting right before selenoneine were detected, which was not present in control worms. However, its presence at concentrations below 1 µg Se/L prevented its identification by HPLC-ESI-Orbitrap-MS. Moreover, trace quantities of a signal co-eluting with SeMet were detected at the L4 stage, but verification with molecular MS was again not possible. The putative SeMet was also found in control worms (Fig. A.1, Appendix), indicating that it might have been an integral part of proteins that was released in the course of sample preparation. It was previously shown that *C. elegans*, analogous to higher organisms, incorporate SeMet into proteins [13]. Altogether, both total Se contents and speciation data revealed that selenoneine was not retained in the worms 48 h post-treatment. Consequently, the protective effect against *t*-BOOH induced RONS formation is unlikely to be caused by the presence of the compound itself. Selenoneine has a more complex, bulkier structure as opposed to other Se species such as selenite, SeMet or MeSeCys. This might be a decisive factor in leading to the absence of protective effects immediately post-treatment. Possibly, the compound is taken up into cells more slowly and/or to a lesser extent compared to smaller Se species, and is mainly present in the worms' intestinal tract at this early time point. Apart from a delayed cellular uptake, differences in metabolism might play a role as well. The formation of highly reactive intermediates such as selenide or methylselenol is considered to be a central step in the activation of redox-active mechanisms by Se species [17]. In general, it is not known if selenoneine is metabolized *via* these intermediates at all, and moreover, if *C. elegans* would be capable of such metabolism. To date, only Se-methylselenoneine has been identified as a putative metabolite of selenoneine in humans [10]. Here, neither Se-methylselenoneine nor any other potential metabolites have been identified following 18 h treatment. In contrast, a comprehensive metabolism of SeMet and MeSeCys was demonstrated after acute (30 min) high-dose exposure in *C. elegans* [15]. Nevertheless, the RONS-attenuating effect observed 48 h post-treatment indicates that selenoneine is not just shuttled through the worms' intestinal tract, but seems to get into the cells and trigger any molecular mechanism, thereby causing this delayed effect. In the previous study, treatment with the Se species had distinct effects on the Nrf2/Skn-1 and FoxO/Daf-16 stress response pathways at the mRNA level [13]. Therefore, the same genes were investigated in the present study. However, their expression levels were not affected by selenoneine, neither directly nor 48 h post-treatment (Fig. A.2, Appendix). Thus, there are no indications on an involvement of the investigated pathways in mediating selenoneine's effects. Elucidating the underlying mechanism clearly requires further studies, since knowledge on selenoneine's mode of action is a necessary attribute for consideration of any possible health benefits in the future. Ergothioneine, the sulfur analogue of selenoneine, has been shown to prevent intracellular RONS accumulation by inhibiting the p38-MAPK signaling cascade, which is activated by H_2O_2, in human neuronal cells [18]. Accordingly, exploring whether selenoneine affects the p38-MAPK pathway as well might be a promising approach.

Conclusion

In the present study, treatment of *C. elegans* with selenoneine did not diminish immediately chemically induced RONS formation, but 48 h post-treatment. Since Se analysis confirmed the uptake and presence of selenoneine after treatment, but failed to demonstrate its retention, the protective effect may not be explained by a radical-scavenging activity of selenoneine. With the experimental design used

in this study, no indications for a direct scavenger function of selenoneine were obtained. Instead, the delayed protection might rather involve the activation of molecular signaling cascades. The underlying mechanism clearly requires further investigation, to better characterize selenoneine's protective effects in the future.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.05.012.

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