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Subacute Oral Toxicity Investigation of Selenium Nanoparticles and Selenite in Rats

Niels Hadrup ^{a,b*}, Katrin Loeschner ^c, Karen Mandrup ^a, Gitte Ravn-Haren ^a, Henrik L. Frandsen ^d, Erik H. Larsen ^c, Henrik R. Lam ^e, Alicja Mortensen ^{a,b}

^a Division for Food, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark; ^b National Research Center For The Working Environment, Copenhagen, Denmark; ^c Division for Food Technology, National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark; ^d Research Group for Analytical Food Chemistry, National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark, ^e Environment and Toxicology, DHI, Hørsholm, Denmark.

Postal addresses, telephone numbers and email addresses:

*Niels Hadrup: National Research Center For The Working Environment, Lersø Parkalle 105, DK-2100 Copenhagen Ø, Denmark, Telephone:+45 39 16 52 14, e-mail: nih@nrcwe.dk

Katrin Loeschner: Division for Food Technology, National Food Institute, Technical University of Denmark, Kemitorvet 201, DK-2800 Kgs. Lyngby, Denmark; e-mail: kals@food.dtu.dk Telephone: +45 35 88 70 29

Karen Mandrup: Division for Food, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kemitorvet 201 2800 Kgs. Lyngby, Denmark; e-mail: karenmandrup@gmail.com

Gitte Ravn-Haren: Division for Food, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kemitorvet 201 2800 Kgs. Lyngby, Denmark; e-mail: girh@food.dtu.dk Telephone: +45 35 88 76 00

Henrik L. Frandsen: Research Group for Analytical Food Chemistry, National Food Institute, Technical University of Denmark, Kemitorvet 202, DK-2800 Kgs. Lyngby, Denmark; e-mail: hlafr@food.dtu.dk Telephone: +45 35 88 75 97

Erik H. Larsen: Division for Food Technology, National Food Institute, Technical University of Denmark, Kemitorvet 201, DK-2800 Kgs. Lyngby, Denmark; e-mail: erik.huusfeldt.larsen@gmail.com

Henrik R. Lam: Environment and Toxicology, DHI, Agern Alle 5, DK-2970 Hørsholm, Denmark; e-mail: hrl@dhigroup.com

Alicja Mortensen: National Research Center For The Working Environment, Lersø Parkalle 105, DK-2100 Copenhagen Ø, Denmark, Telephone:+45 39 16 52 95, e-mail: aam@nrcwe.dk

*For correspondence contact Niels Hadrup (handles correspondence at all stages of refereeing, publication and post-publication), nih@nrcwe.dk , National Research Center For The Working Environment, Lersø Parkalle 105, DK-2100 Copenhagen Ø, Denmark). (At the post publication stage add Alicja Mortensen as an alternative correspondence address aam@nrcwe.dk, National Research Center For The Working Environment, Lersø Parkalle 105, DK-2100 Copenhagen Ø, Denmark)

Subacute Oral Toxicity Investigation of Selenium Nanoparticles and Selenite in Rats

Selenium (Se) nanoparticles have been proposed as food supplements. However, the particle formulation may exert unexpected toxicity. The aim was therefore to compare toxicity of low doses of Se nanoparticles and the dissolved, ionised Se species selenite. Female rats were dosed orally for 28 days with either: 0.05, 0.5 or 4 mg Se/kg body weight (bw)/day as 20 nm Se nanoparticles or 0.05 or 0.5 mg Se/kg bw/day as sodium selenite. Male rats were dosed 4 mg Se/kg bw/day as Se nanoparticles. Body weight and clinical appearance were recorded throughout the experiment. At necropsy, blood samples were taken for haematological and clinical chemistry analyses; organ weights were recorded. At the high dose of Se nanoparticles, overt toxicity occurred and the female animals had to be euthanised prematurely, whereas the male animals were reduced in dose. At all doses of Se nanoparticles and at 0.5 mg Se/kg bw/day as selenite, a lower body weight gain as compared to vehicle occurred. Relative liver weight was increased for both Se formulations at 0.5 mg Se/kg bw/day. Creatinine clearance and urinary pH were affected in some Se dosed groups. There were no effects among dosed groups on brain neurotransmitters or on haematological parameters compared with controls. There were no histological changes in the livers of animals exposed to Se nanoparticles or to selenite. Based on effects on body weight and liver weight, selenium nanoparticles and ionic Se exerted similar toxicity. This suggests that a nanoparticle-specific toxicity of Se did not occur.

Keywords: selenium; nanoparticle; sodium selenite; toxicity; subacute

Introduction

Selenium (Se) is an essential element, necessary for proper cellular function (Levander 1982, Bulteau and Chavatte 2015). Se has been suggested to be effective in cancer prevention among individuals with low Se status (Vinceti *et al.* 2014). Se is a constituent of most food supplements with recommended daily doses of typically 50-100 µg. The tolerable upper intake level is 300 µg/day (SCF 2006). Signs of Se toxicity are e.g. gastrointestinal disorders, irritability, fatigue, hair loss, nail brittleness, dizziness, nausea, concentration difficulties, pulmonary oedema, liver cirrhosis and ultimately mortality (Nelson *et al.* 1943, Sioris *et al.* 1980, Köppel *et al.* 1986, Matoba *et al.* 1986, Hawkes *et al.* 1992, Hunsaker *et al.* 2005, See *et al.* 2006, Spiller and Pfiefer 2007, Sutter *et al.* 2008, MacFarquhar *et al.* 2010, Müller and Desel 2010). The window between intake of Se from food and food supplements and the tolerable upper intake level is narrow. Se nanoparticles (SeNP) are considered to provide a depot formulation of Se through gradual release of dissolved Se from the particle surface. A gradual release formulation has been proposed to be desirable for safer food supplements (Kojouri, Jahanabadi, *et al.* 2012, Skalickova *et al.* 2017). Regarding toxicity, the hypothesis we aimed at investigating was whether SeNP have effects analogous to the molecular selenium species used in food supplements or whether they exert any particle specific toxicity. Particles may disrupt biological structures due to their physical form and catalytic reactions may take place on the particle surface. Thus, it is relevant to assess whether particle specific toxicity occurs for Se, and whether toxicity occurs at higher or lower doses as compared to more traditional ionic Se preparations. This has been addressed in a range of studies. SeNP (20-60 nm in diameter) have been shown to have an acute toxicity seven fold lower than that for selenite in mice based on mortality (Zhang *et al.* 2001). Furthermore, Zhang *et al.* found that oral doses of 6 mg Se/kg bw/day as selenite or as SeNP (20-60 nm) for 12 days to mice suppressed weight gain and induced changes in liver enzymes, with selenite exerting the greatest effect (Zhang *et al.* 2005). Also, at 2 and 4 mg Se/kg bw/day, selenite suppressed growth of mice to a greater extent than SeNP (Zhang *et al.* 2005). Benko *et al.* found selenite and SeNP (100-500 nm in diameter) to be almost equally toxic considering effects on white blood cell counts after administration of 0.004, 0.04 and 0.4 mg Se/kg bw/day for 14 days (Benko *et al.* 2012). In a 13-week rat feeding study, only a slight difference in toxicity between the same two Se preparations was observed based on haematology and organ weight; administered doses were 0.1, 0.2, 0.3 and 0.4 mg Se/kg bw/day as SeNP (20-60 nm in diameter) or selenite and the no observed adverse effect level (NOAEL) was 0.1 mg/kg bw for selenite as compared to NOAEL of 0.2 mg/kg bw for SeNP (Jia *et al.* 2005). Similar toxicity of SeNP and selenite was demonstrated in sheep; an oral dose of 1 mg/kg Se bw/day of SeNPs (smaller than 220 nm) or selenite administered for 10 days resulted in similar effects on serum iron, transferrin expression and increased neutrophil chemotactic activities (Kojouri, Jahanabadi, *et al.* 2012, Kojouri, Sadeghian, *et al.* 2012).

To provide further knowledge on the toxicity of SeNP compared to a dissolved, ionised Se species, we used SeNP 20 nm in diameter and Se in the form of selenite. SeNP was administered at doses ranging from 0.05 (equal to 1.5 fold to the human tolerable

upper intake level, when converted by difference in body surface area according to (Reagan-Shaw *et al.* 2008)(low-dose)) via 0.5 (supra-nutritional dose (mid-dose)) to 4 (high-dose) mg Se/kg bw/day. Selenite was administered to rats at equal low- and mid-doses. The compounds were administered by oral gavage to rats for 28 days. To address general toxicity, standard toxicity parameters were measured. Se toxicity in humans has been reported to include such effects as paraesthesia, memory loss and difficulties of concentration (Clark *et al.* 1996, Sutter *et al.* 2008, Aldosary *et al.* 2012). In order to address whether neurotoxic changes occurred, brain neurotransmitters were measured.

Materials and Methods

SeNP synthesis and characterisation

SeNP were synthesised using a method developed by Zhang *et al.* (Zhang *et al.* 2001) and the synthesis was based on the reaction of selenite and glutathione in the presence of bovine serum albumin (BSA). The Se in the NPs had an oxidation state of 0. The SeNP were characterised with regard to their physico-chemical characteristics before and after the animal experiment. This included size distribution determined by dynamic light scattering (DLS), size and shape by transmission electron microscopy (TEM), and total Se concentration of the suspension by inductively coupled plasma-mass spectrometry before and after filtration of the suspension through 12 kDa filters (Loeschner *et al.* 2014). For DLS, undiluted, 400 mg Se/L, SeNP suspension was directly analysed. For TEM analysis, the suspension was diluted 10 fold with ultrapure water, The mode of the volume-based size distribution by DLS was at around 20 nm (Loeschner *et al.* 2014). The polydispersity index was in the range of 0.174 to 0.236 and the Z-average in the range of 32 to 38 nm. TEM confirmed the determined diameter and showed that the particles had a spherical shape (Loeschner *et al.* 2014). A TEM picture of the SeNP suspension can be seen in Figure 1. The Zeta potential of the SeNP in the undiluted suspension (determined by laser Doppler electrophoresis) was in the range of +10 to 20 mV (pH 4).

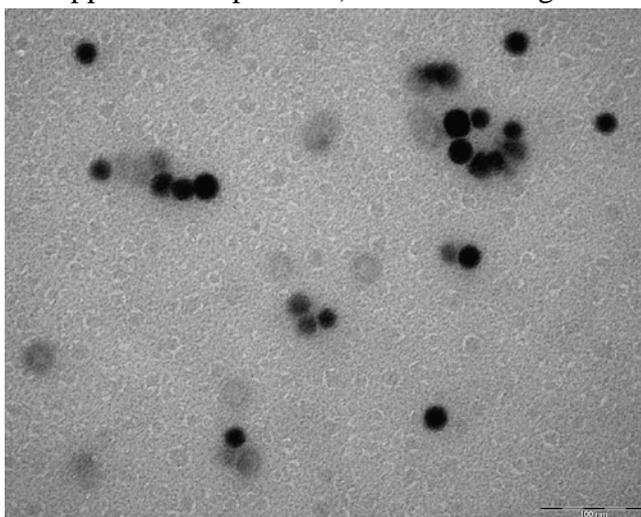


Figure 1. TEM picture of the SeNP suspension

Study design of the 28-day toxicity study

Four weeks old Wistar rats (Wistar Hannover Galas) (Taconic M&B DK-4623, Lille Skensved, Denmark) with specific-pathogen-free health status (SPF), were weight-randomised and allowed to acclimatize for one week. The rats of the same sex were housed two per cage (Macrolon, Techniplats Gazzada S. ar. L., Buguggiate, Italy) with a 12:12-h reversed light/dark cycle with light from 7 p.m. to 7 a.m. The room temperature was 22 ± 1 °C and the relative humidity $55\pm 5\%$. The rats were offered a standard diet (Altromin rat No.1324, Brogård, Gentofte, Denmark) and citric acid acidified tap water (to pH 3), to avoid microbiological contamination of drinking water), both ad libitum. The rats were divided into the following treatment groups: 1. Vehicle-BSA (4g/L) control (8 females and 6 males), 2. SeNP low-dose (0.05 mg Se/kg bw/day) (6 females), 3. SeNP mid-dose (0.5 mg Se/kg bw/day) (6 females), 4. SeNP high-dose (4 mg Se/kg bw/day) (8 females and 6 males), 5. Vehicle-H₂O control (8 females), 6. Selenite low-dose (0.05 mg Se/kg bw/day) (8 females) 7. Selenite mid-dose (0.5 mg Se/kg bw/day) (8 females). The vehicle-BSA group (1) served as control group for the SeNP dosed animals and the vehicle-H₂O group (5) served as control group for selenite dosed animals. The reason for having two control groups is that the SeNP had to be stabilised in BSA; whereas the addition of BSA was not compatible with the selenite. This is because the selenium atom of selenite may passively become incorporated into the BSA molecule in place of its natural content of sulfur in methionine. This would negatively affect the actual selenite concentration in solution before this solution was administered to the animals. At 5 weeks of age dose administration was done by oral gavage at a volume of 10 mL/kg bw/day. Selenite was administered as sodium selenite. Clinical cage-side observations were performed twice daily. Body weight was recorded three times per week. Feed and water intake was recorded at weekly intervals. The feed and water intakes were recorded per cage and thus per two animals. It was thus, when calculating intakes, estimated that the animals in each cage ate and drank equal amounts. At day 14 of dosing the animals were placed individually for 24 hours in metabolism cages for collection of urine and faeces. The urine samples were cooled by sampling on dry-ice and subsequently stored at -80 °C until analyses. During the stay in metabolism cages the rats did not have access to feed. At the end of the 28-day administration period the rats were anaesthetised in O₂/CO₂ and decapitated. Blood for haematology and clinical chemistry was collected from the neck wound in heparin-EDTA tubes. Plasma was prepared by centrifugation at 1000 x g at 4 °C for 10 min. Following necropsy adrenals, liver, spleen, kidneys, testes, epididymis, ovaries, thymus, mesenteric lymph nodes, heart and brain were removed and weighed. Liver specimens from all animals surviving to the terminal sacrifice were fixed in 4% neutral buffered formaldehyde, paraffin-embedded and three micrometre-thick sections were stained with haematoxylin and eosin for histological examination. Brains from all treatment groups were processed for neurotransmitter analysis as described below. The Animal experiments were carried out in accordance with the guidelines stipulated by the National Food Institute's Animal Welfare Committee.

Ethical approval was given by the Danish Animal Experiments Inspectorate (approval number 2004/561-917).

Haematology and clinical biochemistry

White blood cell (WBC) counts, erythrocyte (RBC) counts, haemoglobin concentration (HGB), haematocrit (HCT), platelet (PLT) counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), and mean platelet volume (MPV) were determined in all treatment groups using an Animal Blood Counter Vet (Horiba ABX, Montpellier, France). Blood smears were prepared and stained with May-Grünwald and Giemsa. Differential counting of lymphocytes, monocytes, neutrophil granulocytes, eosinophil granulocytes and basophil granulocytes was done manually by assessing distribution of a total of 100 cells from each animal from the following treatment groups: Vehicle-BSA (females and males), SeNP mid-dose, SeNP high-dose (males) and selenite mid-dose. Differentiation was based on morphologic criteria as described in Bailly and Duprat (Bailly and Duprat 1990). The following clinical biochemical plasma parameters were measured on a Hitachi 912 Automatic Analyzer (Boehringer Ingelheim, Ingelheim am Rhein, Germany) using commercially available kits purchased from Roche/Hitachi (IN, USA): Alanine aminotransferase (ALT) (prod. no. 10851132 216), cholesterol (kit prod. no. 11489232 216), total protein (prod. no.11553836 316), alkaline phosphatase (ALP) (prod. no. 10816388 216), glucose (prod. no. 11447513 216), urea (prod. no. 11489364), creatinine (prod. no. 1104084 216). Urinary glucose, bilirubin, ketones, specific gravity, blood, pH, total protein, urobilinogen, nitrite, and leukocytes were monitored by use of Multistix 10 SG (Prod. no. 2300, Siemens Health Care Diagnostics, NY, USA).

Neurotransmitter analyses

Dopamine, noradrenaline and serotonin were measured as previously described (Hadrup *et al.* 2012). A brief description is as follows, brain tissue was homogenised under ice-cold conditions in 0.32M sucrose, (9 mL/whole brain) using an UltraTurrax T25 homogeniser (IKA, Staufen, Germany). Then the homogenate was de-proteinised using a 1:1 volume of 0.2 M perchloric acid (HClO₄) followed by centrifugation (20 min, 0-4 °C, 10000 xg). For the noradrenaline and dopamine measurements, 3,4-dihydroxybenzylamine (Sigma, D 7012) was added as internal standard. Next, the samples were purified on aluminium oxide (Anton and Sayre 1962) followed by elution with 0.2 M HClO₄. For the analysis of 5-HT, N- ω -methyl-5-HT (Sigma, M 1514) was added as internal standard. The neurotransmitters were measured using high performance liquid chromatography (HPLC) with electrochemical detection. The instrumentation consisted of a Hewlett-Packard Ti-SERIES 1100 liquid chromatograph equipped with an ESA MD-150 analytical column (3 mm ID 15 cm, C₁₈, 5 mm). Separation was achieved with a 5% (v/v) acetonitrile modified 75 mM sodium phosphate buffer (1.7 octenylsulfonic acid, 100 mL triethylamine/L and 25 mM EDTA; pH 3). Signals

were detected using a dual potentiostat electrochemical detector (ESA Model 5200A Coulochem II and ESA Model 5011 high sensitivity analytical cell with an E1 of 150 mV and an E2 of +220 mV; guard cell potential: +350 mV, Chelmsford, MA, USA).

Statistical analyses

The statistical package used was Graph Pad Prism version 4.0 by GraphPad Software (San Diego, CA, USA). Data were tested for normal distribution using the Kolmogorov-Smirnov test. In case of absence of normal distribution data were transformed using the Log_{10} function before a second round of testing for normal distribution. Differences in mean values of normal distributed data were tested using one-way ANOVA with Dunnet's post-test for vehicle-BSA control *vs.* SeNP groups and separately for H₂O control *vs.* selenite groups. A Student's t-test was used to test for differences of mean values for male BSA control *vs.* male SeNP high-dose. In case of absence of normal distribution tests the Kruskal Wallis followed by Dunn's multiple comparisons test was applied. Data were expressed as mean \pm SEM. P values of less than 0.05 were considered statistically significant.

Results

In the SeNP low-dose female group both body weight gain and body weight at the end of the study were lower compared to the vehicle control group. In the SeNP mid-dose female group only body weight gain was lower as compared to the vehicle controls (Figure 2A, Table 1). Regarding selenite female groups, only body weight gain in the mid-dose group was lower (Figure 2B, Table 1). Female rats from the SeNP high-dose group in comparison to the control group did not gain weight (Fig. 2A) during the first week, were less motorically active and local alopecia indicative of hair loss was recorded. Therefore the dose of SeNP was reduced from 4 to 1 mg Se/kg bw/day from day 9 to the end of the experiment. As males receiving SeNP high-dose started presenting the same clinical appearance and low weight gain (Fig 2C) the SeNP dose was reduced from 4 to 1 mg Se/kg bw/day from day 11. Four female rats and two male rats in the high-dose group did not receive SeNPs from day 11 due to the severity of the clinical signs. On day 16, all female rats and two male rats in the SeNP high-dose group were euthanised because of the disturbed clinical condition and necropsy was conducted. The remaining four male rats proceeded on the daily dose of 1 mg Se/kg bw as SeNP until the end of the experiment, and a mean dose received during the dosing period was calculated to 2.2 mg SeNP/kg bw/day based on 11 days with 4 mg SeNP/kg bw and 17 days with 1 mg Se/kg bw as SeNP. Feed and water intake were not affected in any of the groups of rats surviving to the scheduled termination (Table 1).

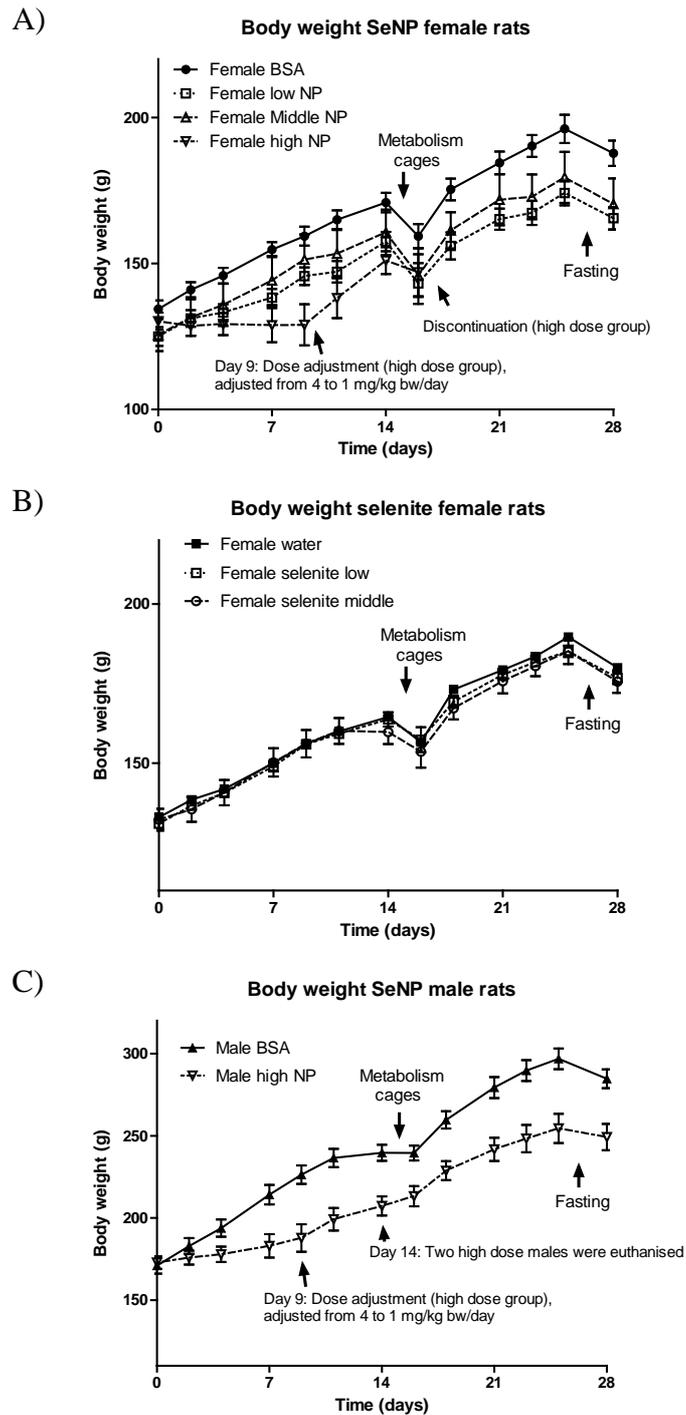


Figure 2. Body weight of rats administered SeNP or selenite. Rats received by gavage SeNP Low-dose (0.05 mg Se/kg bw/day), SeNP mid-dose (0.5 mg Se/kg bw/day) (6 females), SeNP high-dose (4 mg Se/kg bw/day) (Panel A and B), selenite low-dose (0.05 mg Se/kg bw/day) or selenite mid-dose (0.5 mg Se/kg bw/day) (Panel C). Body weight was recorded three times per week. Animals fasted during the last 24 hours of the experimental period, as indicated on graphs. Vehicles were either water or BSA in water. Data are mean \pm SEM. Numerical values and statistics can be found in Table 1.

Haematology values were not affected among the treatment groups (Table 2). Regarding plasma and urine biochemical parameters, only plasma creatinine was decreased in the

SeNP mid-dose female group while creatinine excretion and clearance were increased for high-SeNP dosed male rats. Urine analysis revealed an increase in pH in urine collected from the selenite mid-dose female group and from the SeNP high-dose male rats. No effects were found in the brain concentration of any of the investigated neurotransmitters, dopamine, noradrenaline or 5-HT (Table 3).

Absolute liver weight was decreased in SeNP low-dose females and increased in selenite mid-dose females (Table 4). Relative liver weight was increased in both SeNP high-dose males and SeNP mid-dose females as well as in selenite mid-dose females (Table 5). In SeNP high dose males absolute testes weight as well as absolute and relative weights of the epididymis were decreased (Tables 4 and 5).

Histological examination of the livers from females exposed to low- and mid-doses of either SeNP or selenite and of males from SeNP high-dose group did not reveal any changes as compared to the respective controls.

Discussion

We aimed at assessing the toxic potential of SeNP and whether the toxicity of this formulation differed from that of the dissolved Se-containing molecule, selenite. Overt toxicity occurred at the highest dose. Body weight gain changes, changes in liver weight as well as some biochemical endpoints occurred at mid and low doses. Based on a decrease in body weight gain our data suggested that the lowest SeNP dose (0.05 mg Se/kg bw) was a lowest observed adverse effect level (LOAEL). This suggested that the no observed adverse effect level (NOAEL) for SeNP was even lower. With respect to literature data the toxicity of selenite and of SeNP (100-500 nm) was investigated in mice at doses of 0.004, 0.04 and 0.4 mg Se/kg bw. Equal toxicity was observed as both forms induced higher relative heart weight at lowest dose and decreased the number of white blood cell counts at the two lowest doses (Benko *et al.* 2012). Jia *et al.* administered SeNP or selenite to rats at 0.14 to 0.48 mg Se/kg bw/day, and based on haematology results and organ weight changes, identified a NOAEL of 0.22 mg Se/kg bw/day and 0.14 mg Se/kg bw/day for SeNP and selenite, respectively (Jia *et al.* 2005). Kojouri *et al.* found effects on serum iron, transferrin expression and increased neutrophil chemotactic activities in sheep at 1 mg Se/kg bw/day of SeNP dosed for 10 days (Kojouri *et al.* 2012a; Kojouri *et al.* 2012b). Thus, based on previously published studies on the toxicity of Se, a LOAEL of both SeNP and selenite could be set as low as 0.004 mg Se/kg bw/day given that increased relative heart weight and lowered white blood cells are considered as adverse effects (Benko *et al.* 2012). In our study we found a LOAEL of 0.05 mg Se/kg bw/day, which was based on reduction in body weight gain. This LOAEL was about a factor of 10 higher than that reported by Kojouri *et al.* and could possibly be ascribed to interspecies differences, differences in formulation of SeNP and in sensitivities of endpoints used in the two studies.

Selenite was included to compare the nanoparticle formulation with that of selenite, a commonly used Se species in food supplements. Selenite decreased body weight gain only in animals that received the mid-dose of this Se preparation. At this dose the relative liver weight was also increased as observed also for SeNP. An increase in urinary pH at the mid-dose was a finding not seen for rats dosed with SeNP. Altogether

these data suggest a NOAEL of 0.05 mg Se/kg bw for selenite. We note that this dose is 1.5 fold the human tolerable upper intake level of Se when converted by difference in body surface area; And 10 fold the recommended human high level when considered per body weight. And thus, based on our data, the tolerable upper intake levels could be considered to be too high, as assessment factors of at least 100 would be needed to calculate an acceptable daily intake based on a NOAEL in mg/kg bw.

When comparing the two Se formulations both had effects on body weight gain and relative liver weight, suggesting that they targeted the same endpoints. This is in line with the previously published bio-distribution study of Se in the investigated animals, which demonstrated equal distribution to organs of Se following dosage with SeNPs or selenite (Loeschner *et al.* 2014). More specifically, for Se-containing metabolites in urine, the oral exposure to SeNPs or selenite led to excretion of the same Se-containing metabolites, namely seleno-sugars. This suggested that both forms were metabolised and excreted similarly (Loeschner *et al.* 2014). Moreover, in a previous study based on the same animal experiment we found that both Se formulations displayed similar effects on the general urine metabolome as indicated by partial least squares discriminant analyses, with specific differences in such putative identified metabolites as decanedioic acid and hydroxydecanedioic acid (Hadrup *et al.* 2016). Data from other studies show that ionic forms of Se exert toxicity at lower doses as compared to SeNPs (Zhang *et al.* 2001, 2005, 2008, Jia *et al.* 2005, Wang *et al.* 2007), or that equal or almost equal effects occur following dosage of the two formulations (Benko *et al.* 2012, Kojouri, Jahanabadi, *et al.* 2012, Kojouri, Sadeghian, *et al.* 2012). Combined with results from our study, the available data did not lead to any firm conclusion on whether SeNPs are less or more toxic as compared to dissolved Na selenite, i.e. a specific effect derived from the nanoparticulate form of Se cannot be substantiated.

We note that results from our study and from published results showed that the same toxicity endpoints are affected by both SeNPs and dissolved ionic Se species (Zhang *et al.* 2001, 2005, 2008, Jia *et al.* 2005, Wang *et al.* 2007, Benko *et al.* 2012, Kojouri, Sadeghian, *et al.* 2012, Hadrup *et al.* 2016). An explanation for this could be that SeNP are dissolved in the mammalian body to exert an effect similar to that observed for dissolved selenite.

In conclusion, SeNP and the ionised selenite at the same Se dose administered orally exerted effects on body weight gain and on liver weight in rats. Based on these endpoints, however, the low dose of 0.05 mg Se/kg bw was considered as a LOAEL for SeNP and as a NOAEL for selenite. Overall SeNPs and selenite exerted similar toxicity with respect to endpoints and dose, suggesting that a nanoparticle-specific toxicity did not occur.

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Declaration of interest statement

The authors report no conflict of interest.

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Tables

Table 1. Body weight, water and feed intake in rats treated orally by gavage for 28 days with SeNP or selenite

	Females						Males	
	Vehicle-BSA	SeNP low-dose	SeNP mid-dose	Vehicle-H ₂ O	Selenite low-dose	Selenite mid-dose	Vehicle-BSA	SeNP high-dose
Dose (mg/kg bw/day end)	0 (n=8)	0.05 (n=6)	0.5 (n=6)	0 (n=8)	0.05 (n=8)	0.5 (n=8)	0 (n=6)	4 (n=4 at
Body wt. start (g)	134±8	125±8	126±13	133±8	131±5	132±10	171±12	176±7
Body wt. end (g)	188±12	166±9*	170±22	180±11	177±5	175±10	285±14	249±16 ^{††}
Body wt. gain (g)	54±9	41±5***	45±11**	47±7	46±7	44±4 [§]	114±14	74±16 ^{†††}
Feed intake (g/animal/day)	15±0.3	13±1	14±1	15±0.4	14±0.4	15±1	17±4	17±2
Relative feed intake (g/kg bw/day)	83±2	79±3	81±5	84±6	81±2	83±3	60±11	69±9
Water intake (mL/animal/day)	24±2	20±1	23±3	22±1	22±5	17±2	33±4	27±1
Relative water intake (mL/kg bw/day)	128±8	124±6	133±22	123±11	121±29	97±14	115±8	111±4

Body weight gain was analysed by one-way-ANOVA taking all time points into consideration. *indicates significance by one-way ANOVA followed by Dunnett's post-test for vehicle vs. SeNP 0.05 and SeNP 0.5 mg/kg bw/day in the female group. † indicates significance by t-test vs. male vehicle. Data are mean ± SD.

Table 2. Hematology in rats treated orally by gavage for 28 days with SeNP or selenite

	Females				Males			
	Vehicle-BSA	SeNP low-dose	SeNP mid-dose	Vehicle-H ₂ O	Selenite low-dose	Selenite mid-dose	Vehicle-BSA	SeNP high-dose
Dose (mg/kg bw/day)	0 (n=8)	0.05 (n=6)	0.5 (n=6)	0 (n=8)	0.05 (n=8)	0.5 (n=8)	0 (n=6)	4 (n=4 at end)
WBC (10 ⁹ /l)	3.8±1.4	4.4±1.9	4.4±1.3	5.2±1.1	5.0±1.4	5.2±2.0	4.8±1.1	5.9±1.5
L (%)	85±5		84±7		83±8		84±4	85±5
M (%)	2.3±0.8		2.5±2.4		3.1±1.7		2.5±1.3	2.1±0.5
N (%)	12±5		12±5		13±6		13±4	13±5
E (%)	0.5±0.4		1.3±1.2		1.3±1.3		0.7±0.3	0.6±0.6
B (%)	0.3±0.6		0.1±0.3		0.3±0.5		0.3±0.5	0.0±0.0
RBC (10 ¹² /L)	7.5±0.3	7.7±0.5	7.6±0.4	7.8±0.6	7.7±0.3	7.5±0.5	8.1±0.4	8.0±0.5
HGB (mM)	9.3±0.2	9.6±0.3	9.4±0.5	9.5±0.5	9.4±0.3	9.3±0.4	9.7±0.5	9.2±0.5
HCT (%)	42.2±0.9	43.0±2.6	42.3±2.7	43.2±2.4	42.7±1.4	42.0±2.3	44.7±2.1	43.2±2.9
PLT (10 ⁹ /l)	824±93	664±147	683±132	771±57	780±121	738±83	682±85	661±245
MPV (fL)	6.1±0.1	6.1±0.2	6.0±0.1	6.1±0.2	6.1±0.2	6.1±0.2	6.4±0.2	6.2±0.4
RDW (%)	12.2±0.6	11.7±0.8	11.9±0.2	11.8±0.3	11.9±0.3	12.3±0.4	12.7±0.6	13.3±0.4
MCV (fL)	56.0±1.2	55.8±0.8	56.0±1.3	55.8±1.5	55.5±0.8	56.3±1.3	55.5±1.9	54.3±2.5
MCH (fmol)	19.8±0.7	19.9±0.9	19.9±0.9	19.7±0.8	19.6±0.4	19.9±1.0	19.2±0.8	18.4±0.6
MCHC (mM)	35.3±1.0	35.6±1.3	35.6±1.3	35.3±0.8	35.4±0.7	35.5±1.1	34.6±0.6	34.0±0.5

WBC: White blood cells, RBC: erythrocytes, HGB: haemoglobin concentration, RDW: Red cell distribution width, MPV: Mean platelet volume, Hct: haematocrit, PLT: Platelets, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin Concentration, L: Lymphocytes, M: Monocytes, N: Neutrophil granulocytes, E: Eosinophil granulocytes, B: Basophil granulocytes. For leukocyte distribution 100 cells from each animal were counted. Data are mean ± SD. There were no statistically significant differences.

Table 3. Clinical biochemical and brain neurotransmitter levels in rats treated orally by gavage for 28 days with SeNP or selenite

	Females						Males	
	Vehicle-BSA	SeNP low-dose	SeNP mid-dose	Vehicle-H ₂ O	Selenite low-dose	Selenite mid-dose	Vehicle-BSA	SeNP high-dose
Dose (mg/kg bw/day end)	0 (n=8)	0.05 (n=6)	0.5 (n=6)	0 (n=8)	0.05 (n=8)	0.5 (n=8)	0 (n=6)	4 (n=4 at
ALT (u/L)	53±14	47±17	55±18	53±21	60±22	62±16	69±8	90±23
Cholesterol (mM)	1.8±0.5	1.5±0.4	1.8±0.4	1.4±0.3	1.6±0.1	1.8±0.7	1.8±0.2	2.2±0.5
Protein (g/L)	66±7	66±2	63±2	69±5	70±1	67±2	63±2	65±3
ALP (u/L)	291±97	257±44	242±86	243±88	259±68	235±73	423±130	665±427
Glucose (mM)	6.2±1.5	6.7±1.6	6.1±1.0	6.2±1.6	6.9±0.9	6.4±0.8	7.6±1.7	8.9±1.6
Urea (mM)	21±3	22±4	20±2	20±3	19±4	20±2	20±3	21±4
Plasma creatinine (µM)	36±6	32±2	30±3*	32±3	32±2	30±2	27±3	30±4
Twentyfour hour urine volume (mL/100 g bw)	49±12	56±16	56±20	66±38	59±19	40±20	28±16	32±5
Urine creatinine (µM)	2859±907	2980±1248	3108±2000	2370±760	2276±841	3408±1605	2752±1045	3129±387
Creatinine excretion (µmol/100 g bw/day)	133±26	154±37	146±86	139±90	122±24	113±17	64±7	100±16 ^{###}
Creatinine clearance (mL/100 g bw/day)	3784±865	4818±1073	4823±1813	4281±1546	3206±130	3776±530	2376±288	3430±965 [#]
Urine pH	6.2±0.6	6.1±0.2	6.5±0.4	6.3±0.5	6.2±0.2	6.9±0.6*	6.5±0.3	7.4±0.5 ^α
Brain dopamine (nmol/ g brain tissue)	15.2±2.2	15.2±1.6	13.6±4.0	16.0±3.7	16.1±1.2	16.7±1.5	19.2±1.7	20.2±0.4
Brain noradrenaline (nmol/ g brain tissue)	5.6±1.4	6.9±0.7	7.3±3.6	6.4±1.6	6.2±0.8	6.1±0.6	6.4±0.7	7.2±0.8
Brain 5-HT (nmol/ g brain tissue)	1.7±0.2	1.7±0.1	1.7±0.5	1.8±0.2	1.8±0.2	1.9±0.1	1.7±0.1	1.8±0.02

ALT: alanine aminotransferase, ALP: alkaline phosphatase. *N* is 6-8. Data are mean \pm SD. *designates $p < 0.05$ by Dunn's multiple comparisons test (Kruskal-Wallis) vs. Control, ϖ designates $p < 0.05$ by Mann-Whitney test vs. Male vehicle-BSA. ###designates $p < 0.001$ by students t-test vs. Male vehicle-BSA. #designates $p < 0.005$ by students t-test vs. Male vehicle-BSA.

Table 4. Absolute organ weights of rats treated orally by gavage for 28 days with SeNP or selenite

Dose (mg/kg bw/day)	Females						Males	
	Vehicle-BSA	SeNP low-dose	SeNP mid-dose	Vehicle-H ₂ O	Selenite low-dose	Selenite mid-dose	Vehicle-BSA	SeNP high-dose
	0 (n=8)	0.05 (n=6)	0.5 (n=6)	0 (n=8)	0.05 (n=8)	0.5 (n=8)	0 (n=6)	4 (n=4 at end)
Adrenals (g)	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.06±0.02	0.06±0.01	0.05±0.00
Brain (g)	1.75±0.05	1.68±0.07	1.70±0.08	1.73±0.08	1.73±0.07	1.67±0.08	1.96±0.07	1.85±0.03
Heart (g)	0.64±0.05	0.62±0.03	0.60±0.04	0.61±0.02	0.64±0.06	0.64±0.05	0.92±0.08	0.90±0.16
Kidneys (g)	1.35±0.11	1.24±0.07	1.28±0.10	1.29±0.10	1.31±0.13	1.33±0.13	1.90±0.10	1.86±0.12
Liver (g)	5.40±0.30	4.63±0.20*	5.67±0.75	4.97±0.44	4.91±0.48	6.09±0.50 ^{§§§}	7.61±0.59	9.33±0.88
Lnd. Mesenteric (g)	0.18±0.03	0.18±0.03	0.16±0.05	0.21±0.05	0.20±0.07	0.21±0.04	0.32±0.06	0.27±0.07
Ovaries (g)	0.09±0.01	0.08±0.01	0.09±0.02	0.09±0.01	0.10±0.01	0.09±0.01		
Spleen (g)	0.47±0.06	0.39±0.03*	0.44±0.07	0.43±0.05	0.44±0.04	0.48±0.05	0.64±0.09	0.66±0.13
Testes (g)							3.27±0.39	2.63±0.28 [†]
Epididymis (g)							0.75±0.04	0.57±0.10 ^{††}
Thymus (g)	0.47±0.12	0.39±0.06	0.40±0.07	0.44±0.10	0.45±0.04	0.37±0.05	0.48±0.09	0.34±0.05

N is 4-8. One-way ANOVA with Dunnet's post-test was conducted on female SeNP groups and vehicle. Student's t-test was conducted compare male SeNP with vehicle. Data are mean ± SD. *indicates significance by one-way ANOVA followed by Dunnet's post-test, vehicle vs. SeNP 0.05 and SeNP 0.5 mg/kg bw/day in the female group. [§] indicates significance by one-way ANOVA followed by Dunnet's post-test, vehicle vs. selenite 0.05, and selenite 0.5 mg Se/kg bw/day. [†] indicates significance by t-test vs. male vehicle. Lnd. is an abbreviation for lymph node. For testes and kidneys the reported weights are combined weights of both the right and left organs.

Table 5. Relative organ weights of rats treated orally by gavage for 28 days with SeNP or Selenite

	Females				Males			
	Vehicle-BSA	SeNP low-dose	SeNP mid-dose	Vehicle-H ₂ O	Selenite low-dose	Selenite mid-dose	Vehicle-BSA	SeNP high-dose
Dose (mg/kg bw/day)	0 (n=8)	0.05 (n=6)	0.5 (n=6)	0 (n=8)	0.05 (n=8)	0.5 (n=8)	0 (n=6)	4 (n=4 at end)
(g/100 g bw)								
Adrenals	0.03±0.01	0.04±0.01	0.04±0.00	0.04±0.01	0.03±0.00	0.03±0.01	0.02±0.00	0.02±0.00
Brain	0.93±0.05	1.02±0.07	1.00±0.10	0.96±0.05	0.98±0.05	0.96±0.06	0.69±0.05	0.75±0.04
Heart	0.34±0.04	0.37±0.02	0.35±0.02	0.34±0.02	0.36±0.03	0.36±0.03	0.32±0.02	0.36±0.07
Kidney	0.72±0.04	0.75±0.04	0.76±0.04	0.72±0.06	0.74±0.05	0.76±0.04	0.67±0.04	0.75±0.06
Liver	2.88±0.13	2.81±0.21	3.34±0.26**	2.76±0.21	2.78±0.25	3.48±0.22 ^{§§§}	2.67±0.11	3.77±0.21 ^{†††}
Lnd. Mesenteric	0.10±0.02	0.11±0.02	0.10±0.02	0.11±0.02	0.12±0.04	0.12±0.03	0.11±0.02	0.11±0.03
Ovaries	0.05±0.01	0.05±0.01	0.05±0.01	0.05±0.00	0.05±0.01	0.05±0.01		
Spleen	0.25±0.02	0.24±0.02	0.26±0.04	0.24±0.03	0.25±0.03	0.28±0.03 [§]	0.22±0.03	0.27±0.05
Testes							1.15±0.10	1.06±0.04
Epididymis							0.26±0.02	0.23±0.03 [†]
Thymus	0.25±0.06	0.23±0.03	0.24±0.02	0.24±0.04	0.25±0.02	0.21±0.03	0.17±0.03	0.14±0.01

N is 4-8. One-way ANOVA with Dunnet's post-test was conducted on female SeNP groups and vehicle. Student's t-test was conducted compare male SeNP with vehicle. Data are mean ± SD. *indicates significance by one-way ANOVA followed by Dunnet's post-test, vehicle vs. SeNP 0.05 and SeNP 0.5 mg/kg bw/day in the female group. § indicates significance by one-way ANOVA followed by Dunnet's post-test, vehicle vs. selenite 0.05 and selenite 0.5 mg Se/kg bw/day. † indicates significance by t-test vs. male vehicle. Lnd. is an abbreviation for lymph node. For testes and kidneys the reported weights are combined weights of both the right and left organs.