Selenium Source Impacts Protection of Porcine Jejunal Epithelial Cells from Cadmium-Induced DNA Damage, with Maximum Protection Exhibited with Yeast-Derived Selenium Compounds

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Abstract Selenium (Se) is found in inorganic and organic forms, both of which are commonly used in animal feed supplements. The aim of this study was to determine the impact of the chemical form of Se on its associated ameliorative effects on cadmium (Cd)-induced DNA damage in a porcine model. At a cellular level, Cd mediates free oxygen radical production leading in particular to DNA damage, with consequential mutagenesis and inhibition of DNA replication. In this study, porcine jejunal epithelial cells (IPEC-J2) were pre-incubated for 48 h with one of Se-yeast (Sel-Plex), selenomethionine (Se-M), sodium selenite (Se-Ni) or sodium selenate (Se-Na). The effects of this supplementation on cell viability and DNA damage following cadmium chloride (CdCl$_2$) exposure were subsequently evaluated. IPEC-J2 cells were cultivated throughout in medium supplemented with porcine serum to generate a superior model that recapitulated the porcine gut epithelium. The results illustrated that Se antioxidant effects were both composition- and dose-dependent as evident from cell viability (Alamar Blue and 5-carboxyfluorescein diacetate acetoxymethyl ester) and DNA damage assays (Comet and TUNEL). Both the Se-yeast and Se-M organic species, when used at the European Food Safety Authority guideline levels, had a protective effect against Cd-induced DNA damage in the IPEC-J2 model system whereas for inorganic Se-Ni and Se-Na sources no protective effects were observed and in fact these were shown to enhance the negative effects of Cd-induced DNA damage. It can be concluded that nutritional supplementation with organoselenium may protect porcine gut integrity from damage induced by Cd.

Keywords Selenium · Cadmium · DNA damage · Comet assay · Heavy metal contamination

Introduction

Cadmium (Cd) contamination of animal feed has become a major problem worldwide and is of concern due to its toxic effect and the potential bioaccumulation of Cd through the food chain. Cd is a heavy metal which is naturally present in the environment due to volcanic emissions and the weathering of rocks. However, in recent decades, increased Cd levels have become a concern, primarily due to anthropogenic and industrial activities, the result of which includes contaminated animal feed and feed supplements [1]. The spreading of animal manure has also been shown to contribute to increased levels of Cd in soil, caused directly by contaminated animal feed products [2]. The main route of Cd exposure is through the consumption of contaminated food and water. The European Food Safety Authority (EFSA) has set out regulations regarding permissible levels of Cd in food for human consumption; however, there are currently no regulations relating to acceptable levels of Cd in animal feed. The human body has limited defence mechanisms against Cd, resulting in its bioaccumulation in the food chain. Cd has been shown to induce oxidative stress at a cellular level, with a positive

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correlation noted between Cd dose and the extent of DNA damage [3]. The generation of Cd-induced free radicals has multiple effects on the cell including DNA strand breakage, the generation of mutations and the inhibition of DNA synthesis itself. Cd also affects cell proliferation, cell cycle progression, DNA repair mechanisms and cell differentiation and has also been shown to modify apoptotic pathways (reviewed by [4]). Cd exposure has been reported to lead to nephrotoxicity, osteoporosis and neurotoxicity [5] and to adversely affect gut health and integrity in rats. The gastrointestinal tract is a primary target for Cd-induced damage [6], and a recent study observed tissue injury and a compromised intestinal barrier following Cd exposure [7].

Selenium (Se) is an essential trace element that possesses antioxidant properties and can protect the cell against the harmful effects of reactive oxygen species (ROS), thereby protecting DNA from oxidative damage and consequential disease [8]. Se is a vital dietary component for both humans and animals as it is necessary for the activity of selenoproteins such as glutathione peroxidases (GSH-Px) and thioredoxin reductases, both of which play important roles in detoxification and the function of antioxidants [9]. GSH-Px is responsible for the regulation of hydrogen peroxide levels in the cell. Its activity is significantly influenced by the Se status of the body, suggesting that a decline in Se levels may induce a decline in antioxidant capacity [10]. Se deficiency can lead to poor immune function and increased susceptibility to the damaging effects of ROS, thus promoting cognitive decline and increased risk of mortality in humans. In the Agri-Food industry, low Se status in animals is of great importance as it can lead to white muscle disease, poor reproductive performance and an inability to thrive [11, 12]. Se can exist in various chemical forms, including organic selenomethionine (Se-M) or inorganic selenites and selenates, all of which are commonly utilised as feed additives. Organic and inorganic forms of Se are not metabolised alike [13]. Inorganic selenate is reduced to selenite, which is then non-enzymatically reduced via production of selenodiglutathione (GS-Se-SG) to selenide. Selenide is prone to forming complexes with other feed components leading to the formation of insoluble, unabsorbable complexes which are then excreted, thus significantly reducing Se absorption and bioavailability. The metabolism of organic Se differs to that of inorganic varieties; organic Se compounds are metabolised by an enzymatic process whereby dietary Se is incorporated into protein [9]. One investigation concluded that 98 % of Se from organic sources is absorbed compared to only 84 % for inorganic Se [14]. Several studies have demonstrated that humans absorb and retain Se from organic Se sources more effectively than from inorganic Se compounds ([9] and references therein). Se supplementation has been shown to decrease the risk of prostate, lung, colorectal and bladder cancers due to its protective effect on oxidative DNA damage [15].

Se has been demonstrated to protect against Cd-induced damage in animal models, though results to date are ambiguous. Selenite inhibited Cd-induced damage in chicken kidneys when their feed was co-supplemented with both compounds [16], and the same effect was observed for Cd-induced damage in male rat livers with co-supplementation of selenite and Cd [17]. In an in vitro study using rat hepatocytes, protective effects of selenite were shown to be dependent on Cd concentration and the selenite/Cd ratio, with no inhibition of DNA damage being seen at lower Cd concentrations [18]. Another study showed that a combination of antioxidants, which included Se, had a protective effect against Cd toxicity in rat small intestine [19]. Elsewhere, single supplementation with either Cd or selenite was cytotoxic and genotoxic for rats receiving high pharmacological doses [17] and this effect was even more pronounced for female rats [20]. The potential toxicity of selenite in these models may be problematic, as Se is still widely employed as an animal feedstuff supplement [21]. In pig nutrition in particular, the chemical form of Se has been shown to impact on the animal's growth and overall health [22]. Additionally, organic Se was reported to be superior to selenite regarding the modulation of the DNA repair pathways following lead-induced damage in human liver carcinoma cells [8]. In the case of Se in particular, limitations have been demonstrated regarding the extrapolation of rat models to other models, meaning that the results observed in rat studies cannot automatically be applied to porcine models [23]. It is clear that Se has a role in protecting against DNA damage; however, further work is needed to elucidate this role [24]. To date, the protective effect of Se against Cd-induced damage has not been studied in a porcine model nor have the effects of multiple Se forms been evaluated in that regard. The objective of the present study was to evaluate the potential effects of a range of Se sources on Cd-induced toxicity in a porcine gut epithelial cell model. Here, IPEC-J2 cells were pre-incubated with each of the Se forms Se-yeast (Sel-Plex), selenomethionine (Se-M), sodium selenite (Se-Ni) or sodium selenate (Se-Na) for 48 h, before exposure to $CdCl_2$ for 24 h. The potential cytotoxic effect of $CdCl_2$ was evaluated firstly in the absence of Se, with the nature and extent of the effect of each type of Se form subsequently evaluated relative to this control, using both cell toxicity and DNA damage assays.

Materials and Methods

Cell Culture

IPEC-J2 porcine jejunal epithial cells (IPEC-J2, DSMZ Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's/Ham's Nutrient Mixture F-12 medium (DMEM/Nutrient Mixture F-12 Ham) supplemented with 10 % porcine serum and 1 % penicillin-streptomycin at

37 °C in a humidified 5 % CO_2 atmosphere (Galaxy S CO2 Incubator, Model No: 170–200, RS Biotech Laboratory Equipment Ltd., Irvine, UK). Cells were passaged just prior to confluence every 3 to 4 days following removal with trypsin/EDTA (all cell culture products were from Sigma-Aldrich). All manipulations were done in a class II biological safety cabinet (BioAire Aura 2000 BS; Bioair Instruments, Pavia, Italy). IPEC-J2 cells are an established non-transformed in vitro gastrointestinal model from which results can be extrapolated to the in vivo situation [25, 26]. Here, IPEC-J2 cells were cultivated in the presence of porcine serum (PS) as opposed to conventional foetal bovine serum FBS. This species-specific growth medium supplementation has been shown to promote the growth of IPEC-J2 which is much more similar in terms of cell architecture, morphology, transport functions and trans-epithelial resistance to primary pig jejunocytes [27].

Preparation of Organoselenium Extracts and Selenium Compounds

Forty milligrammes of selenised yeast powder (Sel-Plex®, obtained from Alltech Inc.) was placed in separate 1.5 mL microcentrifuge tubes. Protease enzyme solution (2 mg Protease XIV (Sigma-Aldrich) in 0.5 mL of 10 mM Tris-HCl buffer, pH 7.5)) was added, and tubes were then vortexed gently for 2 min. Samples were ultrasonicated on ice for 25 s at 80 % amplitude (HTU SONI-130 MiniFIER; G. Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany). Ice and MilliQ-H_2O were placed into the microwave carousel and the microcentrifuge tubes were placed into the carousel holder. The extraction programme was run for 15 min at a power output of 30 W and extracted samples were then centrifuged in a microfuge at 14,000 rpm for 3 min. Supernatants were transferred to 15 mL centrifuge tubes. Pellets were washed with MilliQ-H_2O and vortexed until completely resuspended. Samples were re-centrifuged and the supernatants obtained were pooled with the corresponding first supernatants in the 15 mL centrifuge tube. Pooled samples were made up to 15 mL with MilliQ-H_2O and mixed well. Aliquots (2 mL) were then removed for filtration (0.25 μm) and total selenium concentrations were determined by inductively coupled plasma mass spectrometry (ICP/MS; Agilent 7700X; Agilent technologies, Maynooth, Ireland), as described previously [8].

Stock solutions of selenomethionine (Se-M), sodium selenite (Se-Ni) and sodium selenate (Se-Na) were prepared in MilliQ-H_2O, diluted to a final Se concentration of 0.4 ppm in cell culture medium and sterilised by filtration through a 0.2-μm PVDF filter. The Se concentrations chosen for analysis were determined following cell culture toxicity assays (described below) and data is shown in the results section.

Cell Culture Toxicity Assays

Cell viability was evaluated using the fluorogenic indicator dyes Alamar Blue® (AB) (Bio-Source, Invitrogen) and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester; Molecular Probes, Invitrogen) and a multiwell scanning spectrophotometer (Safire II; Tecan Group Ltd., Männedorf, Switzerland), essentially as described elsewhere [28]. A linear relationship between cell number/well and absorption was first established and based on this IPEC-J2 cells were seeded under test conditions at 2×10^4 cells/well of a black 96-well flat bottom plate in the presence or absence of the relevant selenocompounds (Se-yeast (Se-Y), Se-Na, Se-Ni or Se-M) and were incubated at 37 °C for 48 h. The cells were then insulted with 0.5, 0.7 or 1 ppm cadmium chloride ($CdCl_2$) and incubated for a further 24 h at 37 °C. Stock solutions (5 mM) of $CdCl_2$ were prepared in MilliQ-H_2O and then diluted in serum-free growth medium to achieve the desired final concentrations. In brief, after 24 h of incubation with $CdCl_2$, the medium was removed completely and 100 μL/well of the Alamar Blue/CFDA-AM working solution was added. Fluorescence was measured 30 min later at 530 nm excitation/590 nm emission for AB and 485/535 nm for CFDA-AM, respectively, using the multiwell scanning spectrophotometer. Average values of triplicates were calculated and each sample was normalised to its corresponding control.

Comet Assay, DNA Staining and Comet Evaluation

Following pre-incubation with Se compounds and subsequent exposure to $CdCl_2$, IPEC-J2 cells were embedded in agarose comet slides (Trevigen, Gaithersburg, USA) at a concentration of 5×10^5 cells/mL. Cells were then lysed and the DNA was denatured by treatment with 200 mM NaOH for 30 min. Gel electrophoresis was carried out in an alkaline buffer (200 mM NaOH, 1 mM EDTA, pH < 13) at 21 V for 30 min. Gels were then rinsed in MilliQ-H_2O and dehydrated in 70 % ethanol.

Cells were stained following electrophoresis with SYBR Gold (Invitrogen, Bioscience Ltd.) for 30 min at the recommended concentration (Trevigen, Gaithersburg, USA). Cells were observed at ×10 magnification by fluorescent microscopy at excitation/emission wavelengths of 496/522 nm. Results were obtained by collecting data from at least 50 cells per slide for each control/treatment. Cells were analysed and evaluated based on their comet tail appearance. Quantitative analysis of the comet tails was carried out using OpenComet Image J Software Plug-in [29]. Stained cells were also assessed based on previously published grading systems (grades 0–4) with grades 0 and 4 representing those cells with no evident DNA damage and the most DNA damage, respectively [30, 31]. After comet analysis, the individual scores for each of the 50 comets were added and an average value for each treatment was generated. Statistical analysis was performed using one-

way ANOVA; statistical significance was set at $P < 0.05$. In addition to quantitative analysis using the scoring system referred to above, the Comet Tail Moment, Olive Tail Moment, % Tail DNA and Tail Length of each of the comets was also determined using the OpenComet software.

TUNEL Assay

Apoptosis-induced DNA fragmentation was determined by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay which was carried out essentially according to the manufacturer's instructions (Merck Millipore Ireland B. V). Briefly, following Se treatment and incubation with $CdCl_2$, cells were trypsinised and fixed with 4 % (w/v) paraformaldehyde in PBS at a concentration of 1×10^5 cells/mL and stored in 70 % ethanol at −20 °C for 18 h prior to staining. Cells were incubated with TDT enzyme and Brd-UTP antibody for 60 min at 37 °C followed by rinsing. Anti-BrdU-TRITC staining mixture was added to the cells and incubated for 30 min in the dark. Cells were then centrifuged at $300 \times g$ for 7 min; cell pellets were resuspended in 150 μL of rinsing buffer and transferred to a 96-well plate from which data was then acquired on a Guava benchtop flow cytometer (Guava easyCyte 8HT; Merck Millipore, Cork, Ireland). All assays were carried out in triplicate and analyses were performed using the FlowJo software (FlowJo LLC, Ashland, USA). Each Se treatment was analysed relative to its corresponding control and statistical significance was determined by one-way ANOVA.
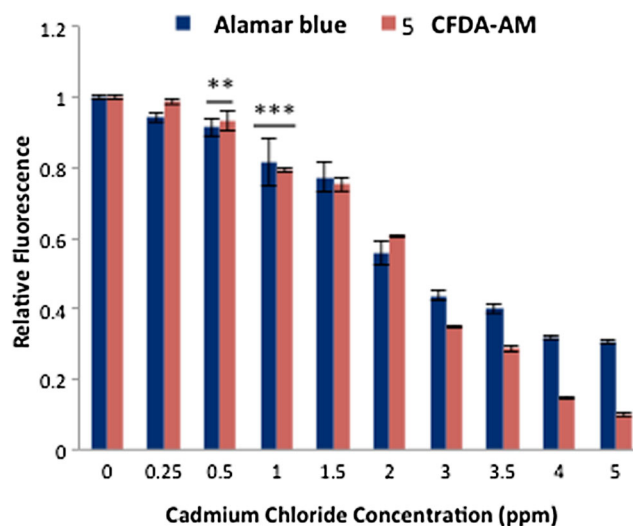
Results

CdCl₂ Is Toxic to IPEC-J2 Cells

The potential cytotoxic effect of $CdCl_2$ on IPEC-J2 cells was analysed over a range of concentrations using an established dual cell viability assay consisting of the fluorogenic indicator dyes Alamar Blue® (AB) and CFDA-AM. AB measures metabolic activity while CFDA-AM reports on membrane integrity. IPEC-J2 cells showed a dose-dependent decrease in cell viability, in both assays, in response to increasing levels of $CdCl_2$ (Fig. 1). Two concentrations of $CdCl_2$ (0.7 and 1 ppm) were chosen for further analysis based on the toxicity curves obtained using both assays.

Effect of Se Sources on IPEC-J2 Viability

The dual viability assay was then used to investigate any potential cytotoxic effects of a range of Se sources on IPEC-J2. The results from both assays showed (Fig. 2a, b) that supplementation of growth medium with Se-Y did not lead to a significant decrease in cell viability at all concentrations tested


Fig. 1 Effect on IPEC-J2 cells of exposure to $CdCl_2$ for 24 h as determined using the combined Alamar Blue/CFDA-AM viability assay. Data are expressed as the mean ± SD of triplicate samples, and the results for each assay are presented as relative to the corresponding unchallenged control (0 ppm Cd) which was set as 1

(up to 1 ppm). Se-Ni had a significant toxic effect ($P < 0.001$), however, at 0.8 ppm. Se-Na and Se-M had no cytotoxic effect at concentrations between 0.2 and 0.8 ppm, but this was followed by a significant loss in cell viability ($P < 0.01$) at concentrations of 1 ppm. Both assays showed the same trend with all Se compounds used. These results concurred with EFSA recommendations for the optimum concentration for Se supplementation, and therefore, 0.4 ppm was used in all subsequent experiments designed to investigate the protective effects of Se against Cd-induced DNA damage.

Modulation of Cd-Induced Cell Viability by Se Sources

The capacity of various Se sources to protect from Cd-induced cell toxicity was next explored. The results from both assays (Fig. 2a, b) showed that there was a significant protective effect at all Cd concentrations used ($P < 0.001$ at 0.5 ppm, $P < 0.05$ at 0.7 ppm and $P < 0.01$ at 0.5 ppm) when cells were pre-incubated with Se-Y relative to Cd-insulted cells that had not received Se supplementation. It was also evident (Fig. 3) that at all concentrations of Cd, there was significantly greater cell viability when Se-Y was used as supplement when compared to Se-Ni and Se-M. Pre-incubation of IPEC-J2 with Se-Na moderated the decrease in cell viability seen due to 0.7 ppm Cd whereas Se-M pre-incubation reduced the level of cell injury induced by 1 ppm Cd. In contrast, significant decreases in cell viability were observed when Cd-treated cells were pre-incubated with Se-Ni (relative to the no-Se control), demonstrating that enhanced Cd-induced cell damage occurred when Se-Ni was used. At all Cd concentrations tested, there were significant decreases in cell viability in the Se-Ni treated cells when compared to Se-Y treated cells
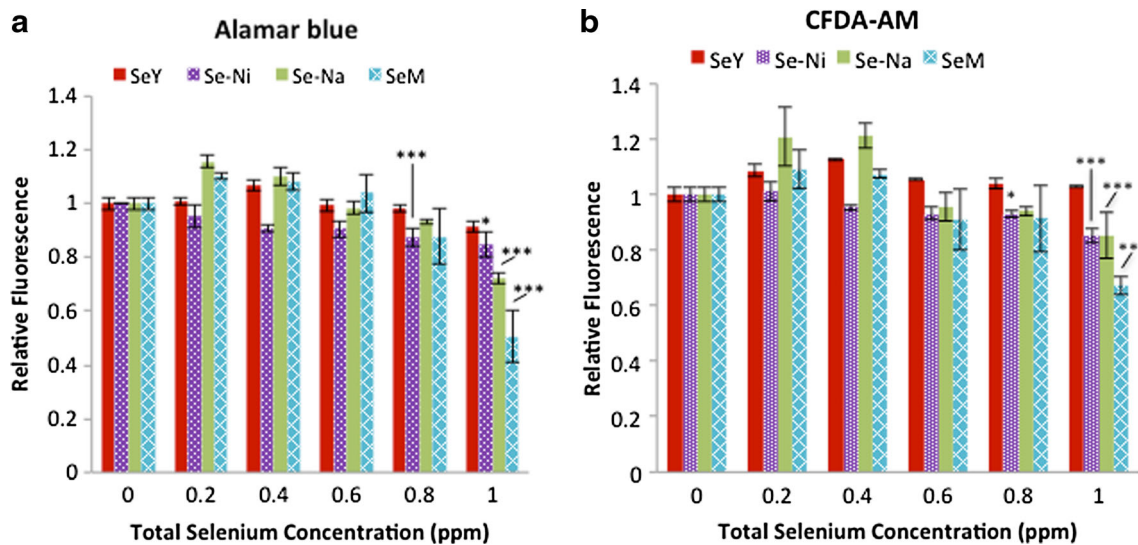
Fig. 2 Effect on IPEC-J2 cells of pre-incubation with a range of concentrations of Se compounds (Se-Y, Se-Ni, Se-Na and Se-M) for 48 h as determined using **a** AB and **b** CFDA-AM assays. Data are expressed as the mean ± SD of triplicate samples, and the results for each assay are presented as relative to the corresponding unchallenged control (0 ppm Se) which was set as 1

($P < 0.001$ at 0.7 ppm Cd, $P < 0.01$ at 1 ppm Cd; data not shown).

DNA Damage Analysis by Comet Assay

The Alkaline Comet assay was employed to assess the extent of Cd-induced single- and double-stranded DNA damage in IPEC-J2 cells which were cultured with or without Se supplementation (Fig. 4). It can be seen that there was significantly less Cd-induced DNA damage (0.7 and 1 ppm Cd) when IPEC-J2 were pre-incubated with Se-Y, relative to no Se supplementation, as evident by lower olive tail moments (Fig. 4b, f), percentage tail DNA (Fig. 4c, g), tail moments (Fig. 4d, h) and tail length values (Fig. 4e, i). In contrast, it can be seen from the same figures that significant increases in Cd-induced DNA damage occurred when Se-Ni was used as supplement, as reflected in comet tail lengths and olive tail moments (0.7 ppm Cd), and percent tail DNA and tail moments (1 ppm Cd) when compared to their respective Cd-treated no-Se controls. This indicated that Se-Ni-treated IPEC-J2 were more sensitive to the effects of Cd, as reflected in increased levels of DNA damage. These experiments also showed that although pre-incubation of cells with Se-M followed by Cd insult (0.7 ppm) led to a decrease in tail moment, increases in tail length and olive tail moments were seen when compared to the corresponding Cd-treated no-Se control. Significant increases were also noted in three of the four Comet parameters, including tail moment, when the 1 ppm Cd/Se-M combination was used implying that Se-M pre-treatment enhanced Cd-induced DNA damage. Pre-incubation of IPEC-J2 with Se-Na resulted in increases to all Comet parameters following exposure to 1 ppm Cd (Fig. 4f–i) relative to Cd-insulted no-Se controls, implying that Se-Na was

potentiating the extent of Cd-induced DNA damage at the concentration of Cd used. Overall, the evidence suggested that pre-incubation of IPEC-J2 cells with Se-Y was more effective at protecting cells from Cd-induced DNA damage, at both Cd concentrations used, than supplementation of growth medium with any of the other Se sources used.

DNA Damage Analysis by TUNEL Assay

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay labels cells that contain nicked DNA and is usually deployed to assess DNA fragmentation that is associated with the onset of programmed cell death (apoptosis). Here, TUNEL was used as a means to further investigate the effect of Cd on Se-supplemented IPEC-J2 cells with the degree of TUNEL-positivity directly reflecting the extent of DNA damage in the cell population under analysis. A significant increase in the TUNEL-positive (apoptotic) cell population was seen following treatment with $CdCl_2$ (0.7 and 1 ppm) indicating the induction of DNA damage and the onset of DNA fragmentation (Fig. 5a, b). Pre-incubation of cells with Se-Y was seen to significantly inhibit the extent of Cd-induced TUNEL-labelling at both Cd concentrations used as reflected in correspondingly increased TUNEL-negative populations. Similarly, pre-incubation with Se-M followed by exposure to Cd correlated with a significant increase in the non-apoptotic population, although not to the same extent as Se-Y when 0.7 ppm Cd was used as insult. In contrast, prior supplementation of cells with either Se-Na or Se-Ni had no protective effect with either concentration of Cd. Overall, this data showed that pre-incubation with either Se-Y or Se-M protected IPEC-J2 cells from Cd-induced DNA fragmentation

a

b
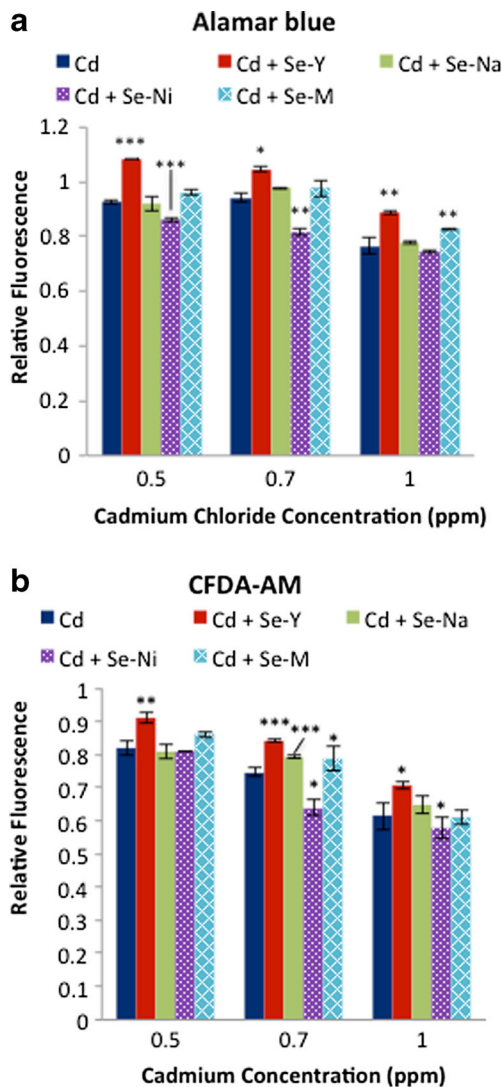
Fig. 3 Effect on IPEC-J2 cells of pre-incubation with Se compounds (0.4 ppm) for 48 h prior to challenge with $CdCl_2$. **a** AB and **b** CFDA-AM assays. The $CdCl_2$ concentrations used are shown beneath each graph. Data are expressed as the mean ± SD of triplicate samples, and the results for each assay are presented as relative to the corresponding Se-treated unchallenged controls (not shown) which were set as 1. Significant differences were determined by one-way ANOVA (denoted as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$) and highlight comparisons between the data points indicated and the corresponding $CdCl_2$-treated no-Se sample

and apoptosis whereas the inorganic Se compounds used did not.

Discussion

IPEC-J2 is increasingly being used as a superior alternative to transformed cell lines, such as Caco-2, for gastrointestinal studies including probiotic screening, feed additive screening and immune and inflammatory studies. It was reasoned that the use of PS (as opposed to FBS) as a growth medium

supplement would further enhance the quality of this model system [27] and that the AB/CFDA-AM dual assay, which measures both mitochondrial enzyme activity and membrane integrity, would be a robust assay combination for assessing cytotoxicity effects. The study presented here represents the first report involving $CdCl_2$ and IPEC-J2. The Cd toxicity data generated here (Fig. 1) concurs with that derived elsewhere with a range of cells lines including HepG2, 1321N1 and HEK 293 using MTT and LDH assays. Measurable decreases in cell viability were observed after 24 h with 0.25 ppm $CdCl_2$ and a significant decrease was seen in all cell lines with 2.5 ppm $CdCl_2$ [32, 33]. Another report investigating Cd-induced damage on LLC-PK$_1$ cells showed that 1 ppm $CdCl_2$ induced apoptosis after 18 h and therefore selected $CdCl_2$ concentrations of 0.5 and 1 ppm for their study [32]. It would appear therefore that Cd-induced cell damage in the porcine model developed here is comparable in terms of cell viability to that observed for multiple human cell lines.

Although Se has been reported to have cytotoxic properties and to prevent oxidative stress in vitro, at higher concentrations, Se can become pro-oxidant and lead to free oxygen radical production and the generation of oxidative stress. It was important therefore to determine the concentration range in which Se supplementation resulted in cytotoxicity. The toxic effects of Se are known to be concentration and composition dependent [34], and both inorganic and organic forms of Se can exhibit pro-oxidant effects leading to the induction of cell apoptosis at high concentrations [35]. The EFSA Food and Feed regulations require that total Se in animal feed products does not exceed 0.2 to 0.4 ppm [36]. In the present study, Se compounds were assayed over a concentration range of 0.2 to 1 ppm (Fig. 2). Se-Y retained an ameliorative effect at all concentrations tested whereas cytotoxicity was detectable for both Se-M and Se-Na at higher concentrations. Se-Ni was seen to promote a modest decrease in cell viability starting as low as 0.4 ppm. Indeed, Se-Ni has been reported elsewhere to promote damage to HepG2 cells after 24 h over a concentration range of 0.25 to 1.25 ppm [37], and to induce cytotoxicity when administered at a concentration of 1 mg/kg in rodent [17] and chicken studies [16]. Importantly, the choice of 0.4 ppm in the present study concurred with the EFSA guidelines for the optimum concentration for Se supplementation. The results obtained here clearly demonstrated that Se-Y did not exhibit any cytotoxic effects at any of the concentrations analysed, and that this was the only form of Se for which this effect was observed. In the porcine model, therefore, it is clear that Se-Y is the safest form of supplementation, up to the maximum concentration analysed, namely 1 ppm.

It was shown that Se-Y was significantly more effective at preventing a decrease in cell viability due to Cd exposure than inorganic Se sources (Fig. 3). That an organic Se source affords more protection correlates with general observations that inorganic Se is generally not as effective at protecting cells

Fig. 4 Determination of DNA damage in IPEC-J2 cells by Comet assay following challenge with CdCl₂. Cells were first pre-incubated for 48 h with various Se sources (0.4 ppm) as indicated underneath each graph, then treated with Cd (0.7 ppm) for a further 24 h prior to analysis by fluorescent microscopy. **a** Representative images of cells that were scored as group 0–IV; the corresponding treatments are given underneath. **b–i** Comet data from various parameters (indicated on the *y*-axis of each graph) as determined using the OpenComet software. The concentration of CdCl₂ used in each case is given underneath each graph. Data expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) and highlight comparisons between the data points indicated and the corresponding CdCl₂-treated no-Se sample
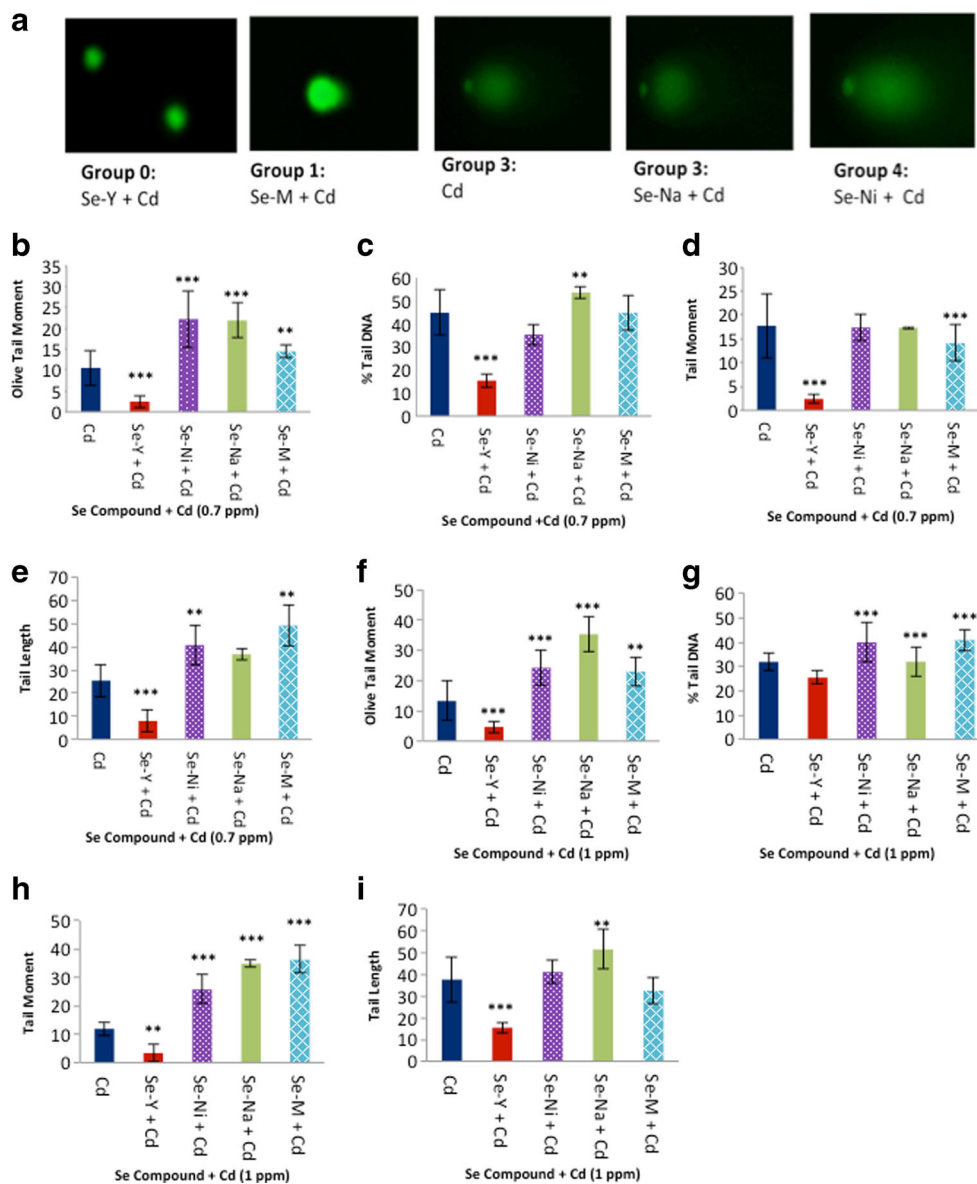
from oxidative stress. This is likely due to the difference in metabolism, absorption and retainment of organic versus inorganic Se compounds [9]. In human cell lines, it was observed that Se-Na was more effective than Se-Ni at promoting resistance to Cd insult. This difference between these inorganic Se sources has been noted elsewhere using the melanoma cell line (HTB 140), human melanocytes and keratinocytes [38]. In one study, a range of cell lines was shown to be more sensitive to treatment with Se-Ni as opposed to Se-Na, as evidenced by decreased cell growth [38]. Another study investigating the effects of Se-M on LNCaP prostate cancer cells reported that Se-M had a significant protective effect in response to oxidative stress [15]. While these studies looked at differences either between inorganic Se sources or explored the effect of organic Se sources, the effects of Se-Y, Se-M, Se-Ni and Se-Na on Cd-induced damage have not been directly

compared in a single study to date. Animal studies have, however, illustrated that organoselenium is more bioavailable and more readily incorporated into biomass than are inorganic Se sources [13]. In the present study, it was clearly demonstrated that organic Se sources inhibited Cd-mediated reductions in cell viability, while inorganic sources promoted these reductions, with Se-Y demonstrating the greatest ameliorative effect in that regard and also being the only Se source that did not lead to decreased cell viability when used alone (in the absence of Cd) as supplement.

The Alkaline Comet assay detects and quantifies both single-stranded and double-stranded DNA breakage. Using this technique, cells are lysed in situ in agarose, electrophoresed and stained with a DNA-binding dye. Following electrophoresis, fragmented DNA migrates out of the nucleoid towards the anode forming a comet shape while undamaged
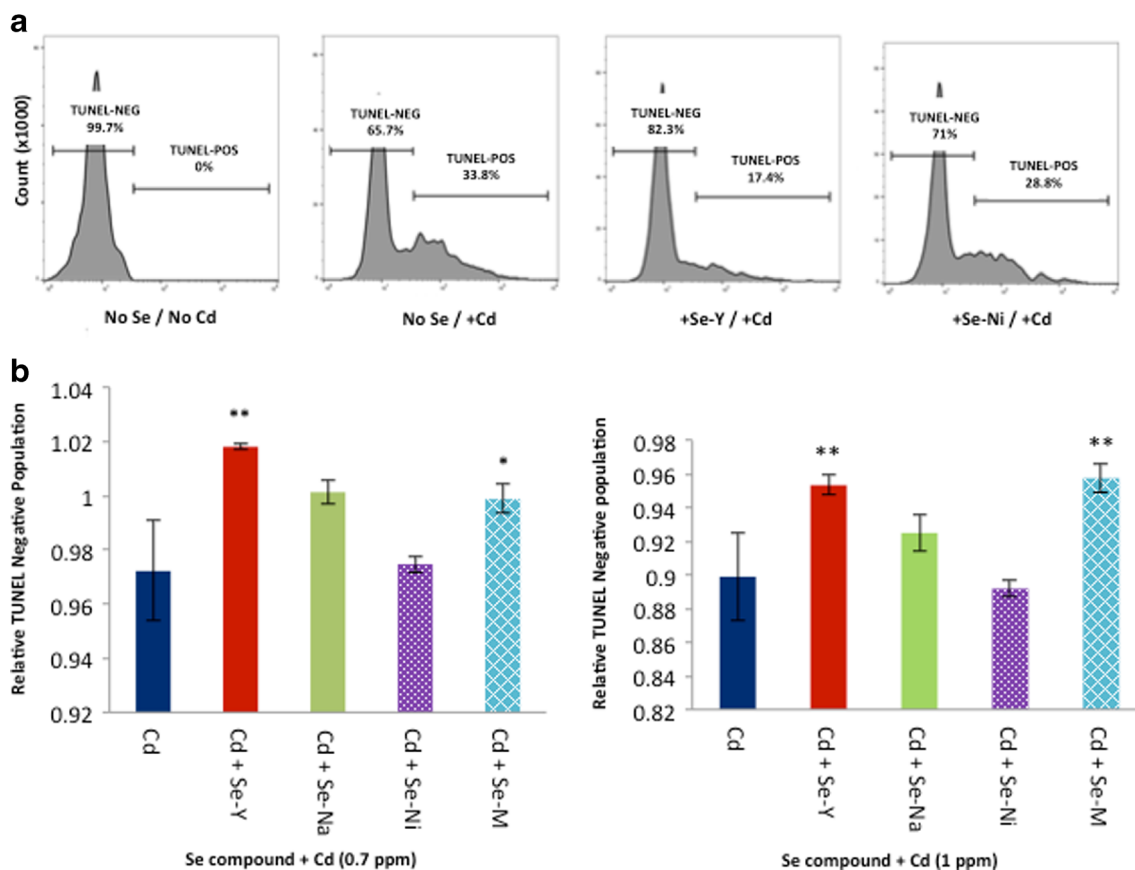
Fig. 5 TUNEL analysis of IPEC-J2 cells following 48 h pre-incubation with Se sources (0.4 ppm) and subsequent exposure to $CdCl_2$ for 24 h. **a** Representative histogram plots of IPEC-J2 cells treated as described underneath each image. TUNEL-negative and TUNEL-positive populations are highlighted in each panel by *bars* (left and right hand sides, respectively). **b**, **c** DNA damage was induced in IPEC-J2 using $CdCl_2$ at 0.7 ppm (**b**) and 1 ppm (**c**) and TUNEL-negative populations are presented as a fraction of the same group in the corresponding $CdCl_2$-treated/no-Se sample. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (***$P < 0.001$) and highlight comparisons made with the corresponding Cd-treated no-Se sample

DNA migrates more slowly under the influence of the electric field [39]. The data derived in the present study using the Comet assay shows that pre-incubation of IPEC-J2 cells with Se-Y afforded significant protection against Cd-induced DNA damage (Fig. 4). The Comet assay results therefore correlate directly with the cell viability results obtained. Se-Y exhibited the greatest ameliorative effect against Cd-induced DNA damage, followed by Se-M, with the inorganic Se sources in fact enhancing the extent of damage due to Cd. Elsewhere, the Comet assay was used to show a comparable effect on lead-induced DNA damage to HepG2 cells following Se pre-treatment, where Se-Y lead to a decrease in lead-induced DNA damage [8]. The same study also showed that Se-Ni pre-treatment resulted in a strong genotoxic effect and a significant increase in DNA strand breakage. The differing effects of Se-Ni and Se-Na supplementation (Fig. 4) is also in agreement with other studies in which the Comet assay was also used to demonstrate enhanced DNA damage promoted by Se-Ni relative to Se-Na in HepG2 and melanoma cells [38, 37].

In the porcine model investigated here, greater levels of DNA damage were noted in Se-M-treated IPEC-J2 cells, relative to those treated with Se-Y. Interestingly, a study in which the digestion and oxidation of different Se compounds was analysed concluded that Se-M concentrations decreased in the small intestine concomitant with the appearance of the oxidation product SeMetO, suggesting that Se-M was prone to targeting by ROS [40]. It has also been reported that the form of Se-M which is used as an additive in animal feed is a synthetic form of L-Se-M. The latter contains D-Se-M as an impurity which is not metabolised efficiently and can build up in organs and tissues leading to toxic effects in the body [41]. In contrast, the Se in Se-Y is highly bioavailable, bioactive and easily absorbed into the bloodstream [42]. Another study which investigated differing gene profiles in response to Se-Y and Se-M using a mouse model suggests that Se-Y comprises several different protein-bound Se compounds in addition to selenomethionine [42]. This indicates that not only is the chemical form of Se important for determining its ameliorative effect, but also the nature of protein-bound Se

complexes. It may therefore be the case that protein-bound forms of Se which lead to increased bioavailability of Se result in a greater protective effect. However, further studies are necessary to explore this possibility.

Here, the TUNEL assay was also used to show that pre-incubation of IPEC-J2 cells inhibited Cd-induced apoptosis-associated DNA fragmentation (Fig. 5). Furthermore, it was evident from the same data that neither Se-Na nor Se-Ni provided any protective effect. These results corroborated the Comet assay data shown above. Elsewhere studies using HepG2 and leukemic HL60 cells showed substantial increases in TUNEL-positive populations following treatment with Se-Ni [37, 43]. It was also observed that pre-incubation of IPEC-J2 with Se-M coincided with a significant decrease in Cd-induced TUNEL-positive cell numbers relative to no-Se controls, although the effect was not as significant as that observed following Se-Y pre-treatment when 0.7 ppm Cd was used. The latter point was not supported from the corresponding Comet assay data however. The fact that Cd-induced apoptosis begins with DNA strand breakage and eventually leads to apoptosis [44] offers a potential explanation for observed higher levels of DNA damage and lower rates of apoptosis as determined by Comet and TUNEL assays, respectively.

In summary, the effects of multiple forms of Se supplementation on cell viability and DNA damage in IPEC-J2 cells following Cd exposure were evaluated. Overall, the data showed that Se protective effects are both composition- and dose-dependent as evident from a range of cell viability and DNA damage assays. It was demonstrated that organic forms of Se exhibited lower levels of cytotoxicity and genotoxicity than inorganic Se forms in this porcine gut epithelial model. At all concentrations analysed, Se-Y did not exhibit any cytotoxic effects and it is postulated that this may be as a result of the nature of the protein-bound Se complexes. It was also demonstrated that organic Se species, when used at the EFSA guideline levels as a growth supplement prior to Cd exposure, have an ameliorative effect against Cd-induced DNA damage in the IPEC-J2 model system whereas inorganic Se sources do not, and can in fact enhance the negative effects of Cd-induced damage. These results are relevant to the agri-food industry and highlight the negative implications of supplementation with inorganic Se forms, as well as the potential for nutritional supplementation in the form of Se-Y to protect gut integrity from damage caused by the environmental contaminant Cd.

References

1. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on cadmium in food. EFSA Journal (2009) 980, 1–139. doi:10.2903/j.efsa.2009.980

2. European Food Safety Authority (2012) Cadmium dietary exposure in the European population. EFSA J 10(1):2551–2588. doi:10.2903/j.efsa.2012.2551

3. Fasanya-Odewumi C, Latinwo LM, Ikediobi CO, Gilliard L, Sponholtz G, Nwoga J, Stino F, Hamilton N, Erdos GW (1998) The genotoxicity and cytotoxicity of dermally-administered cadmium: effects of dermal cadmium administration. Int J Mol Med 1:1001–1007. doi:10.3892/ijmm.1.6.1001

4. Bertin G, Averbeck D (2006) Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88:1549–1559. doi:10.1016/j.biochi.2006.10.001

5. Bampidis VA, Nistor E, Nitas D (2013) Arsenic, cadmium, lead and mercury as undesirable substances in animal feeds. Sci Pap Anim Sci Biotechnol 46:17–22

6. Bergeron PM, Jumarie C (2006) Characterization of cadmium uptake in human intestinal crypt cells HIEC in relation to inorganic metal speciation. Toxicology 219:156–166. doi:10.1016/j.tox.2005.11.016

7. Ninkov M, Popov A, Demenesku J, Mirkov I, Mileusnic D, Petrovic A, Grigorov I, Zolotarevski L, Tolinacki M, Kataranovski D, Brceski I, Kataranovski M (2015) Toxicity of oral cadmium intake: impact on gut immunity. Toxicol Lett 237:89–99. doi:10.1016/j.toxlet.2015.06.002

8. McKelvey SM, Horgan KA, Murphy RA (2014) Chemical form of selenium differentially influences DNA repair pathways following exposure to lead nitrate. J Trace Elem Med Biol. doi:10.1016/j.jtemb.2014.06.005

9. Zeng H, Combs GF (2008) Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. J Nutr Biochem 19:1–7. doi:10.1016/j.jnutbio.2007.02.005

10. Brummer M, Hayes S, Dawson KA, Lawrence LM (2013) Measures of antioxidant status of the horse in response to selenium depletion and repletion. J Anim Sci 91:2158–2168. doi:10.2527/jas.2012-5794

11. Ward P, Connolly C, Murphy R (2013) Accelerated determination of selenomethionine in selenized yeast: validation of analytical method. Biol Trace Elem Res 151:446–450. doi:10.1007/s12011-012-9571-x

12. Ishii M, Ogata H, Shimizu H, Takeuchi Y, Nozawa T, Yamamoto Y, Okamoto T, Shimamura T, Utsumi A, Jitsukawa T, Endo M (2002) Effects of vitamin E and selenium administration on pregnant, heavy draft mares on placental retention time and reproductive performance and on white muscle disease in their foals. J Equine Vet Sci 22:213–220. doi:10.1053/jevs.2002.34302

13. Galbraith ML, Vorachek WR, Estill CT, Whanger PD, Bobe G, Davis TZ, Hall JA (2015) Rumen microorganisms decrease bioavailability of inorganic selenium supplements. Biol Trace Elem Res 171:338–343. doi:10.1007/s12011-015-0560-8

14. O'Dell BL, Sunde RA 1997 Handbook of nutritionally essential minerals. New York: Marcel Dekker, Inc; 275–334. ISBN 0–8247–9312-9

15. De Rosa V, Erkekoğlu P, Forestier A, Favier A, Hincal F, Diamond AM, Douki TRW (2012) Low doses of selenium specifically stimulate the repair of oxidative DNA damage in LNCaP prostate cancer cells. Free Radic Res 46:105–116. doi:10.3109/10715762.2011.647009

16. Liu L, Yang B, Cheng Y, Lin H (2015) Ameliorative effects of selenium on cadmium-induced oxidative stress and endoplasmic

reticulum stress in the chicken kidney. Biol Trace Elem Res 167: 308–319. doi:10.1007/s12011-015-0314-7

17. Jabeen F, Chaudhry AS (2011) Effects of cadmium chloride and sodium selenite alone or in combination on the liver of male Sprague-Dawley rats assessed by different assays. Biol Trace Elem Res 143:1077–1090. doi:10.1007/s12011-010-8946-0

18. Yu, RA, Chen X (2004) Effect of selenium on rat hepatocellular DNA damage induced by cadmium in vitro. Chinese J Prev Med 38:29–32. doi: DOI:. 10.3760/J: ISSN: 0253–9624.2004.01.009

19. Bolkent S, Koyuturk M, Bulan OK, Tunali S, Yanardag R, Tabakoglu AO (2007) The effects of combined alpha-tocopherol, ascorbic acid, and selenium against cadmium toxicity in rat intestine. J Environ Pathol Toxicol Oncol 26:21–27

20. Forrester LW, Latinwo LM, Fasanya-Odewumi C, Ikediobi C, Abazinge MD, Mbuya O, Nwoga J (2000) Comparative studies of cadmium-induced single strand breaks in female and male rats and the ameliorative effect of selenium. Int J Mol Med 6:449–501. doi:10.3892/ijmm.6.4.449

21. Schrauzer GN, Surai PF (2009) Selenium in human and animal nutrition: resolved and unresolved issues. A partly historical treatise in commemoration of the fiftieth anniversary of the discovery of the biological essentiality of selenium, dedicated to the memory of Klaus Schwarz (1914-1978). Crit Rev Biotechnol 29:2–9. doi:10.1080/07388550902728261

22. Surai PF, Fisinin VI (2016) Selenium in sow nutrition. Anim Feed Sci Technol 211:18–30. doi:10.1016/j.anifeedsci.2015.11.006

23. Hamilton-Koch W, Snyder RD, Lavelle JM (1985) Metal-induced DNA damage and repair in human diploid fibroblasts and Chinese hamster ovary cells. Chem Biol Interact 59:17–28. doi:10.1016/S0009-2797(86)80052-7

24. Bera S, De Rosa V, Rachidi W, Diamond AM (2013) Does a role for selenium in DNA damage repair explain apparent controversies in its use in chemoprevention? Mutagenesis 28:127–134. doi:10.1093/mutage/ges064

25. Geens MM, Niewold TA (2011) Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization. Cytotechnology 63:415–423. doi:10.1007/s10616-011-9362-9

26. Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, Jores J, Tachu B, Kleta S, Blikslager A, Tedin K, Wieler LH (2006) Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol 125:293–305. doi:10.1007/s00418-005-0067-z

27. Zakrzewski SS, Richter JF, Krug SM, Jebautzke B, Lee IF, Rieger J, Sachtleben M, Bondzio A, Schulzke JD, Fromm M, Günzel D (2013) Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. PLoS One 8:e79643. doi:10.1371/journal.pone.0079643

28. Bopp SK, Lettieri T (2008) Comparison of four different colorimetric and fluorometric cytotoxity assays in a zebrafish liver cell line. BMC Pharmacol 11:1–11. doi:10.1186/1471-2210-8-8

29. Gyori BM, Venkatachalam G, Thiagarajan PS, Hsu D, Clement M (2014) Redox Biology OpenComet : an automated tool for comet assay image analysis. Redox Biol 2:457–465. doi:10.1016/j.redox.2013.12.020

30. Azqueta A, Collins AR (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol 87:949–968. doi:10.1007/s00204-013-1070-0

31. Nandhakumar S, Parasuraman S, Shanmugam MM, Rao KR, Chand P, Bhat BV (2011) Evaluation of DNA damage using single-cell gel electrophoresis (comet assay). J Pharmacol Pharmacother 2(2):107–111. doi:10.4103/0976-500X.81903

32. Zhou YJ, Zhang SP, Liu CW, Cai YQ (2009) The protection of selenium on ROS mediated-apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK(1) cells. Toxicol in Vitro 23:288–294. doi:10.1016/j.tiv.2008.12.009

33. Lawal AO, Ellis E (2010) Differential sensitivity and responsiveness of three human cell lines HepG2 , 1321N1 and HEK 293 to cadmium. J Toxicol Sci 35:465–478. doi:10.2131/jts.35.465

34. Letavayova L, Vlckova V, Brozmanova J (2006) Selenium : from cancer prevention to DNA damage. Toxicology 227:1–14. doi:10.1016/j.tox.2006.07.017

35. Fernandes AP, Gandin V (2015) Selenium compounds as therapeutic agents in cancer. Biochim Biophys Acta 1850:1642–1660. doi:10.1016/j.bbagen.2014.10.008

36. EFSA panel on additives and products or substances used in animal feed (FEEDAP) (2011) Scientific opinion on safety and efficacy of Sel-Plex ® (organic form of selenium produced by *Saccharomyces cerevisiae* CNCM I-3060) for all species. 9:1–52. doi: 10.2903/j.efsa.2011.2110.

37. Shen HM, Yang CF, Ong CN (1999) Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG2 cells. Int J Cancer 81:820–828. doi:10.1002/(SICI)1097-0215(19990531)81:5<820::AID-IJC25>3.0.CO;2-F

38. Bandura L, Drukala J, Wolnicka-Glubisz A, Björnstedt M, Korohoda W (2005) Differential effects of selenite and selenate on human melanocytes, keratinocytes and melanoma cells. Biochem Cell Biol 211:196–211. doi:10.1139/O04-130

39. Speit G, Hartmann A (2006) The comet assay: a sensitive genotoxicity test for the detection of DNA damage and repair. Methods Mol Biol 314:275–286. doi:10.1385/1-59259-973-7:275

40. Lavu RVS, Van de Wiele T, Pratti VL, Tack F, Du Laing G (2016) Selenium bioaccessibility in stomach, small intestine and colon: comparison between pure Se compounds, Se-enriched food crops and food supplements. Food Chem 197:382–387. doi:10.1016/j.foodchem.2015.08.001

41. Devos C, Sandra K, Sandra P (2002) Capillary gas chromatography inductively coupled plasma mass spectrometry (CGC-ICPMS) for the enantiomeric analysis of D,L-selenomethionine in food supplements and urine. J Pharm Biomed Anal 27:507–514. doi:10.1016/S0731-7085(01)00576-3

42. Barger JL, Kayo T, Pugh TD, Vann JA, Power R, Dawson K, Weindruch R, Prolla TA (2012) Gene expression profiling reveals differential effects of sodium selenite, selenomethionine, and yeast-derived selenium in the mouse. Genes Nutr 7:155–165. doi:10.1007/s12263-011-0243-9

43. Shi K, Jiang Q, Li Z, Shan L, Li F, An J, Yang Y, Xu C (2013) Sodium selenite alters microtubule assembly and induces apoptosis in vitro and in vivo. J Hematol Oncol 6:1–9. doi:10.1186/1756-8722-6-7

44. Gu P, Bernhard D (2016) Cadmium overkill: autophagy, apoptosis and necrosis signalling in endothelial cells exposed to cadmium. Cell Mol Life Sci 73:1699–1713. doi:10.1007/s00018-015-2094-9