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Selenium toxicosis assessment (*in vivo* and *in vitro*) and the protective role of vitamin B12 in male quail (*Coturnix Coturnix*)

M.A. Gad*, S.M. Abd El-Twab

Department of Zoology, Faculty of Science, Beni-Suief University, Salah Salem Street, Beni-Suief 62514, Egypt

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ABSTRACT

The present study was undertaken to elucidate the toxicity induced by sodium selenite in male quail through *in vivo* and *in vitro* studies and the role played by vitamin B12 in alleviating selenium toxicity. Administration of selenite orally for 1 month induced hepatic oxidative damage. Selenite decreased body weight gain and increased relative liver weight. Selenite reduced hemoglobin and iron concentrations and elevated total bilirubin concentration. Serum transaminases and alkaline phosphatase activities were increased in selenium-intoxicated quails. Total protein concentration was decreased associated with the appearance of prealbumin fraction, an increased γ-globulin and a decreased α- and β-globulins. The highest level of selenium was found in liver followed by kidney, testis, faeces and blood. Supplementation of vitamin B12 orally for 1 month simultaneously with selenite caused less marked biological alteration in the investigated parameters. *In vitro* study using isolated quail hepatocytes incubated with sodium selenite showed a dose-dependent response for toxicity markers. These results suggest that selenosis can be reduced by vitamin B12 supplementation.

1. Introduction

Description of toxicity from selenium ingestion dates back to the time of Marco Polo in the late 13th century (Yang et al., 1983). Selenium (Se) is an element that has been of environmental concern primarily in arid environments undergoing irrigation for agricultural purposes (Presser and Ohlendorf, 1987; Skorupa, 1998). The form and amount of selenium ingested varies by the geochemistry of the soil in which food is grown (Reid et al., 2004). Adverse effects on fish and birds have been noted as a result of dietary accumulation of organoselenides such as the amino acid conjugates selenomethionine and selenocysteine, which are derived from transformations occurring within the food chain (Maier and Knight, 1994). Moreover, both inorganic sodium selenite and selenate are approved by the Food and Drug Administration (FDA, 1987) for inclusion in the diets of farm animals. The selenite form is, however, more commonly used because of its lower relative cost. Although the contribution of indigenous selenium from grain sources to livestock diets is not regulated by FDA, other sources of inorganic selenium salts have not been approved and cannot be incorporated into diet formulations. It has been reported that both sodium salt forms of selenium (i.e., selenite and selenate) are of potential danger to humans because of their rapid water solubility, hence toxic responses might occur when in direct contact with skin and mucous membranes (Echevarria et al., 1988).

Acute selenosis occurs after an extremely high dietary intake (≥20 mg/kg b.w.) or injection (≥1.65 mg/kg b.w.) of selenium (Mahan and Moxon, 1984). They added that chronic selenosis occurs after consuming diets containing 5–20 ppm selenium over a longer time period. The mechanism of the cytotoxicity of selenite and other redoxing selenium compounds is believed to derive from its pro-oxidant ability to catalyze the oxidation of thiols and to produce superoxide simultaneously (Spallholz, 1994; Stewart et al., 1999). In addition, it may be referred to DNA oxidative damage and fragmentation in cells (Wilson et al., 1992; Stewart et al., 1999), liver cirrhosis and anaemia (Kim and Mahan, 2001), and cellular apoptosis (Stewart et al., 1997). Also, when isolated hepatocytes were incubated with selenite (30–100 μM) *in vitro*, a depletion of thiols including reduced glutathione (GSH) was observed (Anundi et al., 1984). Other studies revealed that selenite stimulates lipid peroxidation *in vitro* and *in vivo* (Csallany et al., 1984; Hoffman, 2002). Spallholz (1994) concluded that selenium toxicity manifests itself when oxidative damage exceeds antioxidant defenses or the ability of animals to form selenoproteins, selenoethers or elemental selenium.

Selenium accumulated in soft tissues approximately in proportion to the amount ingested in the diet and the type of tissue

* Corresponding author. Tel.: +20 100332745; fax: +20 82 2334551.
E-mail address: Maamoun_gad@yahoo.com (M.A. Gad).

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(Echevarria et al., 1988; Albers et al., 1996). Therefore, critical body burden thresholds for selenium have been recommended as a method to assess ecological risk to susceptible wildlife (Hamilton, 2002).

Chemical toxicity *in vivo* is ultimately a process that occurs at the cellular level and thus can be studied *in vitro*. The ability to obtain an approximate prediction of the *in vivo* toxic dose by means of a simple *in vitro* test could reduce the suffering caused during *in vivo* testing by permitting the selection of selenium doses which would be better tolerated by the animals and this might, in turn, also reduce the overall number of animals used.

Vitamin B12 is a coenzyme for various metabolic functions, including fat and carbohydrate metabolism and protein synthesis (Jacobs and Wood, 2003). Methylcobalamin is an active form of vitamin B12, which is required for the remethylation of homocysteine to methionine catalyzed by the methionine synthetase (Marsh, 1999).

The current study was undertaken to elucidate the toxicity induced by sodium selenite in male quail through *in vivo* and *in vitro* studies via (1) the assessment of the alterations in both serum biochemical and hepatic oxidative stress parameters and electrophoretic pattern of serum proteins induced by sodium selenite *in vivo*, (2) the modification of *in vitro* toxicity markers by selenium in isolated quail hepatocytes. Also, it aimed to study the role of vitamin B12 in alleviating the negative effects of sodium selenite intoxication in quail.

2. Materials and methods

2.1. Experimental design

Fifty-four adult male quails (*Coturnix coturnix)* weighing 170–190 g were housed in well ventilated woody cages (80 cm × 120 cm large enough for 12 birds) at normal atmospheric temperature (26 ± 2 °C) as well as under 14 h of light per day, and acclimatized for 2 weeks. The birds were fed a standard diet for chickens (a complete pelleted feed mixture for poultry of all age groups) and received clean, fresh water *ad libitum*.

In study I (*in vivo*), 48 birds were randomly assigned to four groups (12 per group). The 1st group (selenium) received selenium as sodium selenite (Na_2SeO_3, Code No.10102-18-8, Aldrich Chemical Company, USA) at a dose of 6 mg/kg b.w. (Hoffman, 2002) orally through gavage for 1 month (4 days/week). The 2nd group (vitamin B12) was given vitamin B12 (Code No V6629, Sigma–Aldrich Company) at a dose of 0.3 mg/kg b.w. (Couce et al., 1991) by gastric intubation for 1 month (4 days/week). The 3rd group (selenium + vitamin B12) received sodium selenite followed by vitamin B12 at the two tested doses used for group one and two, respectively. The 4th group (control) received distilled water orally at an equal volume to that used for the treated groups along the experimental periods. In study II (*in vitro*), six acclimated adult male quail were used for studying toxicity markers, in isolated quail hepatocytes, mediated by sodium selenite.

Body weight gain was calculated from the difference between the initial weight at the beginning and the final weight at the end of the experiment. Birds of different groups were sacrificed after 2 and 4 weeks of the experiment, and the blood was collected in heparinized tubes for the estimation of hemoglobin content. Blood samples were also collected in separate tubes and serum was separated for selenium residue and biochemical investigations, and electrophoretic pattern identification. Livers were excised immediately, rinsed in cold saline and stored at −20 °C where one portion was used for various biochemical investigations, and the other portion, with collected samples of kidney, testes and faeces, were used for selenium residue estimation.

2.2. Chemicals

All chemicals and reagents used in the present study were of the highest purity available, and were purchased from Sigma (St. Louis, MO, USA), and Merck (Darmstadt, Germany) Companies.

2.3. Biochemical studies

The levels of hepatic reduced glutathione (GSH) and total thiol (T. thiol) were determined by the methods of Beutler et al. (1963) and Koster et al. (1986), respectively. The activity of hepatic superoxide dismutase (SOD) was measured according to the method of (Marklund and Marklund, 1974). Liver catalase (CAT) was determined according to the technique of Cohen et al. (1970) and the results were expressed in terms of the first-order reaction rate constant (*K*). Heme peroxidases (myeloperoxidase and eosinophil peroxidase) (P_x) activity in the liver was assayed according to the method of Kar and Mishra (1976) with some modifications using pyrogallol as a substrate. Briefly, to 50 μl of supernatant, 150 μl phosphate buffer solution (pH 6.8), 16 μl 2% pyrogallol and 7.7 μl 50 μM H_2O_2 were added. After exactly 30 min the density of purpurogallin colour was measured against the blank at 420 nm. The peroxidase enzyme activity was expressed in absorbance (A). Thiobarbituric acid reactive substances (TBARS) indicative of lipid peroxidation was determined by the thiobarbituric acid reaction in the liver tissue according to the method of Preuss et al. (1998).

Hemoglobin concentration was estimated according to the method of Lucky (1977) using reagent kits purchased from Randox Company (UK). Iron concentration was assessed according to the method of Henry (1974) using reagent kits obtained from Sclavo Diagnostics Company (Italy). Bilirubin concentration was determined according to the method of Tietz (1986) using reagent kits provided by Bio-Adwic (Egypt). Both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed according to the method of Reitman and Frankel (1957) using reagent kits purchased from Randox Company (UK). Gamma-glutamyltransferase (γ-GT) activity was assessed according to the method of Beleta and Gella (1990) using reagent kits obtained from Biosystems Company (Spain). Alkaline phosphatase (ALP) activity was determined according to the method described by Belfield and Goldberg (1971) using reagent kits obtained from Bio-Merieux Chemical Company (France). Total proteins (TP) concentration was measured according to the method of Henry (1964) using reagent kits obtained from Diamond Diagnostic Company (Egypt). Moreover, the protein profile (Albumin, Globulin and Globulin fractions) was further assessed electrophoretically in serum according to the method of Kohin (1958). The strips of the cellulose acetate electrophoretic pattern were scanned and the absolute values of different protein fractions (bands) were calculated according to the values of total protein. Albumin/globulin (A/G) ratio was calculated according to the equation described by Doumas et al. (1971).

2.4. Selenium residue analysis

Samples of serum, liver, kidney, testes and faeces were analyzed for selenium residue at the end of the experiment according to the method of Schlenk et al. (2003) using Graphite Frnace-Atomic Absorption Spectrometer (GFAAS).

2.5. In vitro cytotoxicity assessment

In vitro cytotoxicity assessment was carried out according to the methods described by Repetto et al. (2001) and Wang et al. (2004) with some modifications.

Quail was anaesthetized by thiopental (20 mg/kg; EMEA, 1999), followed by disinfection of the skin, opening the abdomen, exposing the portal vein, and heparin sodium (100–150 U) was injected into the portal vein. When the liver was isolated at liver hilus, it was first perfused with calcium and magnesium-free Hank's buffer at 80–100 ml/min for 10–15 min. The liver was then perfused with 0.5 g/l collagenase solution at 50–70 ml/min for 10 min. Perfusion systems were kept at 37–38 °C. After perfusion, the liver capsule was incised, the thick fibrous connective tissue was discarded, and cell suspensions were harvested. The hepatocyte suspension was washed 2–3 times with phosphate buffered saline (PBS, pH 7.4).

Cells were resuspended in Leibowitz L-15 medium and centrifuged (50 g for 10 min) in a 90% percoll solution to improve the separation of viable and non-viable cells, as described by Kreamer et al. (1986). The cell viability, as determined by trypan blue exclusion, was typically greater than 95%.

Hepatocytes were suspended in Eagle's medium containing 10% (v/v) fetal calf serum, 100 U penicillin G/ml and 100 μg streptomycin/ml, plated at a density of 10^4 cells/well in 96-well tissue culture plates, and allowed to attach to the substratum for 2 h, then the medium was changed to remove non-attached cells.

Following the attachment period, hepatocytes were immediately incubated in the growing solution containing the test chemical (sodium selenite) at different concentrations (50, 100 and 200 μM) and incubated for 2, 4 and 8 h at 30 °C in a humid atmosphere of 5% CO_2: 95% air. The growing medium without the test chemical served as control.

At the end of the tested periods, supernatants were collected for the determination of toxicity markers (LDH leakage, thiobarbituric acid reactive substances, glutathione content, glucose consumption and total protein concentration). LDH was assessed according to the method of Rec. GSCC (1970) using reagent kits purchased from Diamond Diagnostics Chemical Company (Egypt). Glucose concentration was determined according to the method of Trinder (1969) using reagent kits obtained from Spinreact Company (Spain).

2.6. Statistical analysis

The Statistical Package for the Social Sciences (SPSS for windows Version 11.0; SPSS Inc., Chicago) was used to test the normality of the present data to define which test should be used. The results expressed as mean ± S.D. were analyzed using one way analysis of variance (ANOVA) followed by LSD computations to compare various groups with each other (Rao and Blane, 1995).